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Physiological and Morphological Responses to Osmotic Stress in Barley (*Hordeum vulgare* L.)

by

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This thesis contains one literature review chapter and five main research chapters. One section in the literature review chapter (Chapter 2) has been published as a book chapter. Results described in the three research chapters (Chapter 3, Chapter 4 and Chapter 5) have been published in three different journals.

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Candidate was the primary author and contributed 70% to conduct the bulk experiments and wrote the paper draft. Author 1 and Author 2 contributed to the conception and design the experiments, data interception and took the leading role in writing. Author 3 and Author 4 contributed to provide logistic support for this experiment, writing, proof-reading and revision of the manuscript.

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Hasanuzzaman M, Shabala L, Brodribb TJ, Zhou M, Corkrey R, Shabala S (2018) Factors determining stomatal and non-stomatal (residual) transpiration and their contribution towards salinity tolerance in contrasting barley genotypes. *Environ Exp Bot* **153**:10-20

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Conference Papers

Hasanuzzaman M, Shabala L, Brodribb TJ, Zhou M, Shabala S (Poster presentation) "Assessing the suitability of various screening methods as a proxy for drought tolerance in barley". InterDrought-V, Hyderabad, India, 21-25 February, 2017

Hasanuzzaman M, Davies NW, Shabala L, Brodribb TJ, Zhou M, Shabala S (Oral presentation) "Residual transpiration determines differential salinity stress

tolerance in barley". 18th Australian Barley Technical Symposium, Hobart, Tasmania, Australia, 3-6 September, 2017

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List of Abbreviations

ABA, abscisic acid

ANOVA, analysis of variance

APX, ascorbate peroxidase

ATP, adenosine triphosphate

BADH, betaine aldehyde dehydrogenase

CAT, catalase

CDK, cyclin-dependent kinase

CMO, choline monooxygenase

CRD, complete random design

DB, glycinebetaine

DH, doubled haploid

DW, dry weight

EC, electrical conductance

EPP, epidermal patterning factor

FAO, Food and Agriculture organisation of the United Nations

F_v/F_m, maximal quantum efficiency of PS II

FW, fresh weight,

GCMS, gas chromatography-mass spectrometry

Gs, stomatal conductance

MIFE, microelectrode ion flux estimation system

NAPDH, nicotinamide adenine dinucleotide phosphate

PEG, polyethylene glycol

PPO, polyphenol oxidase

PSII, photosystem II

PVC, polyvinyl chloride

QTL, quantitative trait locus

RH, relative humidity,

ROS, reactive oxygen species

RT, residual transpiration

RWC, relative water content

SD, stomatal density

SDI, salinity damage index

SE, standard error

SEM, scanning electron microscope

SOD, superoxide dismutase

SOS1, salt overly sensitive 1, Na^+/H^+ antiporter

SOS2, salt overly sensitive 2, a serine/threonine protein kinase

SOS3, salt overly sensitive 3, a myristoylated calcium binding protein

TIA, Tasmanian Institute of Agriculture

V-ATPase, vacuolar type H^+-ATPase

WC, water content

WUE, water use efficiency

Abstract

Global food production must increase by at least 70% to feed an additional 2.4 billion people by 2050 while world agriculture combats major biotic and abiotic stresses brought by the climate change. Salinity and drought are arguably the two most severe abiotic environmental stresses among these that affect agricultural crop production globally. Therefore, future food security cannot be achieved without a major breakthrough in crop breeding for salinity and drought stress tolerance. The early responses of plants to drought and salinity are similar, as both stresses result in a cellular water deficit. This causes a decrease of the cytosolic and cell vacuolar volumes that inhibit plant growth and productivity. Plants deal with osmotic stress by employing a range of biochemical, morphological and physiological mechanisms. However, it has become clear in recent years that osmotic stress tolerance is highly multifaceted traits, determined by a number of sub-traits, of which the efficient control of stomatal and non-stomatal (residual) transpiration are the most crucial components to increase the efficiency of CO_2 assimilation. To attain the overall goal of producing robust salinity and drought tolerant cultivars, it is important to quantify the relative contribution of stomatal and residual transpiration in the overall osmotic stress tolerance and to identify their components as a trait determining osmotic stress tolerance. Hence, the major aim of this PhD project was to investigate the stomatal and non-stomatal transpiration and their relative contributions toward salinity and drought stress tolerance, plus the overall plant performance under salinity and drought conditions in contrasting large number of barley genotypes. In this thesis the following specific objectives were addressed: (i) to establish the importance of the residual transpiration as a component of salinity tolerance mechanism; (ii) to reveal the role of cuticular waxes as a determinant of the residual transpiration; (iii) to assess the suitability of different physiological and morphological traits as a proxy for drought tolerance; (iv) to understand the selective physiological and morphological traits contributing to drought tolerance in a large number of barley genotypes.

Four barley (*Hordeum vulgare* L.) genotypes contrasting in their salinity tolerance were used to evaluate the relationship between residual transpiration to the overall

salt tolerance, and also investigated what role of cuticular waxes play in this process. Results revealed that leaf osmolality, osmotic potential, leaf water potential and the amount of total cuticular wax are involved in controlling residual transpiration from barley leaves surface under well irrigated conditions. A significant and negative relationship between the amount of primary alcohols and the residual transpiration implies that some cuticular wax constituents also act as a water barrier on plant leaf surface and thus contribute to salinity stress tolerance. It is suggested that residual transpiration could be a fundamental mechanism by which plant can reduce water use during stress.

We compared different physiological measures of drought stress in six barley genotypes subjected to different drought treatments under glasshouse conditions to find a convenient, reproducible, reliable and rapid screening method to be used a proxy for drought tolerance for a large number of barley genotypes. Genotypes were evaluated by measuring transpiration rate, quantum yield of PSII (chlorophyll fluorescence F_v/F_m ratio), chlorophyll content, dry biomass and shoot water content under drought stress. The transpiration rate and leaf growth/death were quantified after rewatering. In another experiment, the same genotypes were evaluated by applying 18% (w/v) polyethylene glycol (PEG) to germinating seeds grown in paper rolls to induce osmotic stress, using relative root and shoot lengths as a measure of osmotic stress tolerance. The results suggested that transpiration measurements at the recovery stage could be the most sensitive method for evaluating the stress sensitivity of different genotypes. Chlorophyll fluorescence (F_v/F_m ratio) of dark-adapted leaves could be recommended as a suitable proxy for screening tolerance of water stress. Measuring relative root growth rate (length) using PEG-treated paper roll-grown seedlings also seems to be a highly suitable and promising method for screening a large number of genotypes in breeding programs.

Based on our previous work, eighty barley genotypes of different geographical origin and contrasting in salinity stress tolerance were grown under glasshouse conditions and exposed to high salinity stress (300 mM NaCl) for four weeks to investigate the relationship between leaf gas exchange, tissue ionic relations, and overall plant salinity tolerance. Four weeks after the treatment commenced,

stomatal conductance, stomatal density, residual transpiration, chlorophyll content, leaf sap Na^+, K^+, Cl^- concentration and leaf sap osmolality were measured. Responses to salinity stress differed greatly among the genotypes. The overall salinity tolerance significantly correlated with leaf Na^+ content, osmolality, stomatal density and the residual transpiration. The results suggested that increasing stomatal density as well as minimization of the residual transpiration may be a promising way of improving water use efficiency and increase salinity tolerance in barley. Our data also showed that residual transpiration is strongly affected by the number of stomatal pores on the leaf surface.

To identify the desirable morphological and physiological traits that confer drought stress tolerance, we screened eighty barley genotypes collected from different geographical locations and contrasting in drought stress tolerance. Plants were exposed to continuous drought stress by withholding irrigation for four weeks under glasshouse conditions. Also, root length of the same genotypes was measured from stress-affected plants growing hydroponically. The drought tolerance was scored 30 days after the drought stress commenced based on the degree of leaf damage, fresh and dry biomass and relative water content. These characteristics were related to stomatal conductance, stomatal density, residual transpiration and leaf sap Na^+, K^+, Cl^- contents measured in control (irrigated) plants. Responses to drought stress differed significantly among the genotypes. The overall drought tolerance was significantly correlated with relative water content, stomatal conductance and leaf Na^+ and K^+ concentration. No significant correlations between drought tolerance and root length of 6-day-old seedling, stomatal density, residual transpiration and leaf sap Cl^- content were found. Taking together, these results suggest that drought tolerant genotypes have lower stomatal conductance, and lower water content, and lower Na^+, K^+ and Cl^- contents in their tissue under control conditions than the drought sensitive ones.

In conclusion, the overall studies suggested that residual transpiration is associated with salinity stress tolerance. The total amounts of cuticular wax or cuticular wax components, specifically primary alcohol, act as a water barrier to reduce water loss through the plant leaf surface. Increasing stomatal density and reducing residual transpiration are the promising way of improving water use efficiency under salinity stress contributing to increased salinity tolerance in

barley. Interestingly, residual transpiration strongly correlated with the number of stomatal pores on the leaf surface. Measuring chlorophyll fluorescence of dark adapted leaves (F_v/F_m ratio) is recommended as an efficient and promising method for screening a large number of genotypes in breeding programs. Plants with lower stomatal density and stomatal conductance under irrigated conditions showed higher drought tolerance under water deficit conditions. Barley plants with lower Na^+, K^+ and Cl^- concentration in their tissue showed greater tolerance under drought stress which revealed that tolerant genotypes are more dependent on organic osmolytes than the inorganic ions for osmotic adjustment under drought stress conditions.

Chapter 1. General Introduction

1.1 Background

1.1.1 Salinity and drought stress as issues

The growth rate of crop yields has reduced by 1-2% per decade over the past century (Gourdji et al., 2013). Crop yields could decrease by 11% along with an increase in food price by 20% in 2050 due to climate change (Wiebe et al., 2015). Drought and salinity are arguably the most severe environmental stresses driven by the current trends in the global warming and climate change affecting crop growth and productivity worldwide. Drought and salinity affect more than 10% of cultivated land, and rapidly increasing on a global scale, reducing average yields for most major crops by more than 50% (Bartels and Sunkar, 2005). Over a third of the earth including world's best food production zones will be affected by drought in the next 30-90 years (Dai, 2011; Dai, 2013). On the other hand, the salinity stress affected area is increasing daily, cutting crop yields by 20-50% in many regions in the world (Shrivastava and Kumar, 2015). About 20% (ca 62 million ha) of total cultivated irrigated land is adversely affected by salinity globally, costing approximately $27.3 billion each year (Qadir et al., 2014). Thus, salinity and drought issues will remain the key threats to the global food production in the 21st century. At the same time, the world population is projected to increase by more than 9.7 billion in 2050 and increase further 11.2 billion by 2100; this will require an increase in food production by 70% by 2050 to meet this demand (Alexandratos and Bruinsma, 2012). Therefore, future food security cannot be achieved without a major breakthrough in crop breeding for salinity and drought stress tolerance.

This study has been focused on barley (*Hordeum vulgare* L.), one of the major cereals which has the capacity to adapt to salinity and drought stress. According to statistics published by FAO, worldwide barley production in 2016-2017 amounted to approximately 148.03 million tons and was ranked fourth among cereals, after wheat, rice, and maize. Barley is used as a staple food in different regions in the world, where it faces salinity stress and drought stress, during their growth episodes that adversely affect growth and productivity. Although barley is relatively salt and drought tolerant crop compared to the other cereals, a 70-80%

reduction in grain yield is observed at severe drought stress (Samarah et al., 2009) and 20-50% decline of yield at high salinity (Hammami et al., 2017). Hence, a comprehensive understanding of mechanisms of salinity and drought tolerance in physiological and morphological level and breeding for salt and drought-tolerance barley genotypes are critical to recover the yield penalty of barley under salinity and drought stress conditions.

1.1.2 Plant adaptation to osmotic stress induced by salinity and drought

The initial responses of plants to drought and salinity stress are similar. Both stresses contribute to "physiological drought" which induces osmotic stress by lowering soil water potential in the root zone and inhibiting plant growth and productivity (Munns, 2002). The osmotic effect of both stresses can be observed immediately after stress is induced and dominates for a few weeks (Munns and Tester, 2008). At the cellular level the osmotic stress causes cell dehydration by removal of water from the cell cytoplasm into the extracellular space, thus decreasing cytosolic and vacuolar volume (Bartels and Sunkar, 2005). Osmotic stress induced by salinity and drought may lead to various physiological symptoms, such as denaturation of cytosolic and organelle proteins, compromised membrane integrity, nutrient imbalance, production of reactive oxygen species (ROS), reduced rate of cell division and cell expansion in growing tissue, stomatal closure and decreased photosynthetic activity (Forni et al., 2017; Munns and Tester, 2008).

Plants have developed several strategies to deal with osmotic stress by combining physiological, anatomical, biochemical and metabolic aspects. Different osmotic stress tolerance or defence mechanisms are involved in enabling plants to cope with the adverse effect of salinity and drought. Such mechanisms include a network of ion transport, *de novo* synthesis or accumulation of compatible solutes, enzymatic or non-enzymatic antioxidants and detoxifying ROS systems, hormonal regulation and transcription factors. Osmotic stress-responsive genes are activated under stress conditions resulting in the production of important metabolic proteins and the regulation of the downstream genes for signal transduction to protect the cells from stress. The majority of salinity and drought-induced genes function in damage restriction or repair; this includes late embryogenesis abundant (LEA)

proteins, osmotin, antifreeze proteins, chaperones and ubiquitination-related enzymes (Forni et al., 2017). Different proteins and Ca^{2+} kinases and phosphatases are involved as signal transducers in osmotic stress signalling and transduction to down-stream gene transcription such as mitogen-activated protein kinase (MAPK), SNF1/AMP-activated protein kinases, calcium dependent protein kinase (CDPK), receptor protein kinase, calcineurin B-like proteins (CBLs), CBL-interacting protein kinases (CIPKs), protein phosphatases and proteinases (Forni et al., 2017). A large number of transcription factors are involved in the stress sensory pathway to tolerance mechanisms and participate in transcriptional regulation of osmotic stress tolerance. This includes basic region leucine zipper (bZIP), homeodomain leucine zipper proteins (HD-ZIP), Zn-finger proteins, apetala2/ ethylene response factor (AP2/ERF), MYB-like proteins, MYC-like proteins and CDT-1 (Bartels and Sunkar, 2005).

Plants have evolved enzymatic and non-enzymatic antioxidant defence systems to overwhelm the harmful effects of increased ROS either by producing antioxidant compounds or by increasing the activity of enzymes for ROS scavenging. The enhancement of the activity of enzymes such as guaiacol peroxidase, superoxide dismutase, catalase, glutathione peroxidases, ascorbate peroxidase, glutathione reductase, monodehydroascorbate reductase, dehydroascorbate reductase and glutathione reductase have been reported under osmotic stress conditions (Ahmad et al., 2010; Gill and Tuteja, 2010). The non-enzymatic antioxidants such as ascorbic acid, α-tocopherols, glutathione, carotenoids, phenolic compounds and alkaloids are also involved in maintaining ROS below the threshold level that causes cellular injuries (Ahmad et al., 2009). The accumulation or *de novo* synthesis of organic (proline, glycine-betaine) and inorganic (Na^+, K^+, Cl^-) osmolytes are involved in mediating osmotic adjustment by overcoming osmotic stress and re-establishing cellular homeostasis that help the cells to maintain their hydrated state providing resistance against drought and cellular dehydration (Puniran-Hartley et al., 2014). Thus, it has been clear that osmotic stress tolerance is a highly multifaceted trait which is determined by a number of sub-traits and genes. Therefore, to achieve the overall goal of development of a truly tolerant cultivar, different sub-traits should be emphasised in a highly compatible and complementary manner.

Amongst all the sub-traits for osmotic stress tolerance, efficient control of stomatal and non-stomatal transpiration is the most crucial to conserve water under osmotic stress conditions. When physiological water deficit under osmotic stress conditions is increased enough to induce complete or partial closure stomata, the way of water loss through the leaf surface to atmosphere are across the cuticle and through leaky stomatal closure. This process is referred to as a residual transpiration. Under such conditions, the fitness and survival capacity of plants depends on the limitation of water loss through leaf cuticle with minimum stomatal aperture. The genotypes which have the capacity to reduce the water diffusion from the cuticle could potentially conserve more water in tissue to survive under osmotic stress conditions. Thus, residual transpiration could be a component of osmotic stress tolerance. This study investigated the role of the residual transpiration in osmotic stress tolerance in barley using a large number of barley genotypes contrasting in drought and salinity stress tolerance. While residual transpiration could be regulated by the characteristics of the leaf surface, total amount of cuticular wax, physical properties and orientation of cuticular wax, plant water relations and the composition of cuticular wax (Jetter et al., 2007; Vasantha et al., 2015), it is still unclear whether residual transpiration is correlated with the total amount of cuticular wax or the chemical constituents of the cuticular wax or leaf water status (Schuster et al., 2016; Zeisler and Schreiber, 2016). Answering this question was one of the aims of this study.

Plants may exercise transpirational control of leaf by the regulation of stomatal characteristics (stomatal density) and stomatal conductance under stressful environmental conditions. Stomatal density could be related to non-stomatal transpiration through the leaf. Arguably, the manipulation of stomatal density could be a fundamental mechanism by which plant can regulate water use efficiency. It was found that water use efficiency increased by ~ 20% in *Arabidopsis* mutants by stomatal manipulation as a result of the overexpression of *EPF2* (epidermal patterning factor) (Franks et al., 2015). On the other hand, stomatal density and stomatal conductance are correlated with each other (Franks and Beerling, 2009). However, to the best of our knowledge no study has linked highly salinity stress-induced changes in stomatal density with salinity stress tolerance in barley using sufficient large number of genotypes. Is it good for a

plant to have fewer stomata, to reduce transpiration? Or is it better to have many partially closed stomata? What are the factors affecting stomatal characteristics under high salinity stress conditions? Answering these questions was another objective of this study.

Due to the physiological complexity of drought stress, finding a convenient and reliable phenotyping method for screening a large number of barley genotypes to identify the drought-tolerant germplasms is challenging, regardless of whether the trial is done in the field, laboratory or pot. Several physiological, morphological and agronomical traits have been used as proxies for drought stress tolerance in the past. These include transpiration rate, stomatal conductance, stomatal density, F_v/F_m ratio, chlorophyll content, relative water content, root length and biomass. One of the aims of this study was a critical evaluation of these physiological and agronomical traits as proxies for drought tolerance.

1.2 Objectives and research aim

This project was aimed to provide answers to the above questions. To achieve this aim a large number of barley genotypes were screened for osmotic stress tolerance under controlled glasshouse conditions. The correlations between different physiological and morphological traits and the overall salinity and drought stress tolerance were quantified, and the importance of residual transpiration control as a trait predicting salinity and drought tolerance was established.

The following specific objectives were addressed:

(1) Evaluate the relative contribution of residual transpiration to the overall salinity tolerance in barley and investigate the role of cuticular waxes as a determinant of the residual transpiration

Residual transpiration could be a potentially useful mechanism for improving plant performance under stress environmental conditions. Reduction of residual transpiration under osmotic stress conditions while stomata are completely or partially closed is a promising way of improving water use efficiency. Deposition of cuticular wax on leaf surface acts as a barrier of diffusion of water loss across the impermeable cuticle under stress conditions. Arguably, cuticular wax is negatively correlated with residual transpiration. However, it is still not clear

whether the residual transpiration is related to the total amount of cuticular wax or it is related to the specific constituents of cuticular wax. In this part, we investigated the effect of residual transpiration on overall salinity tolerance and the relationship of residual transpiration to plant water relations, and cuticular wax load at three different leaf positions under irrigated conditions using two highly salt tolerant and two salt sensitive barley genotypes.

(2) Develop suitable screening methods for drought tolerance in barley

The availability of convenient, robust, simple, reproducible, reliable and rapid screening protocols is crucially important for plant breeders to efficient phenotype plant germplasm and developing better stress-adapted genotypes. Another critical question is what agronomical and physiological key traits are most suitable to be used as a proxy for drought tolerance? The aim of this part was to develop a simple, reliable and reproducible way of imposing of the drought stress on plants, and to assess the suitability of various physiological and agronomical traits as proxies for barley drought tolerance for high-throughput screening of barley germplasm.

(3) Determine the factors affecting stomatal and various physiological indices for salinity tolerance in barley genotypes

The main objectives of this part were to answer the following questions: what are the factors affecting stomatal characteristics under high salinity stress conditions? Is stomatal density correlated with salinity tolerance? Should these traits be targeted in the barley breeding programmes? The overall objectives of this part were to identify the relative contributions of stomatal density and to quantify their components under higher salinity stress conditions.

(4) Understand the physiological and morphological traits contributing to drought tolerance in barley genotypes

Different plants have developed multiple mechanisms through integrated morphological, anatomical and physiological responses to cope with drought stress. This includes modification of the root system; regulation of stomatal characteristics; controlling stomatal and non-stomatal transpiration. Thus, the

desirable key traits of drought tolerance mechanisms were identified in a series of glasshouse experiments as a part of this study.

1.3 Outline of the chapters

Chapter 2: Literature review about physiological and morphological mechanisms mediating plant tolerance to osmotic stress: balancing tolerance and productivity

Chapter 3: Residual transpiration as a component of salinity stress tolerance mechanism: a case study for barley

Chapter 4: Assessing the suitability of various screening methods as a proxy for drought tolerance in barley

Chapter 5: Factors determining stomatal and non-stomatal (residual) transpiration and their contribution towards salinity tolerance in contrasting barley genotypes

Chapter 6: Understanding physiological and morphological traits contributing to drought tolerance in barley

Chapter 7: General discussion and conclusions

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Chapter 2. Review of Literature

Physiological and morphological mechanisms mediating plant tolerance to osmotic stress: balancing tolerance and productivity[1]

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[1]This chapter has been published as a book chapter: Hasanuzzaman M, Zhou M, Shabala S (2018) Physiological and Morphological Mechanisms Mediating Plant Tolerance to Osmotic Stress: Balancing Tolerance and Productivity. In: Climate Change and Crop Production: Foundations for Agroecosystem Resilience, ed. Benkeblia N, CRC press, Taylor & Francis Group

Chapter 3. Assessing suitability of various screening methods as a proxy for drought tolerance in barley[2]

Abstract

Plant breeders are in the need for a convenient, reproducible, reliable and rapid screening method to be used as a proxy for drought tolerance for a large number of genotypes. Addressing this need, we compared different physiological measures of stress in six barley (*Hordeum vulgare* L.) genotypes subjected to different drought treatments under glasshouse conditions. Genotypes were evaluated by measuring transpiration rate, quantum yield of PSII (chlorophyll fluorescence F_v/F_m ratio), SPAD chlorophyll meter reading, dry biomass and shoot water content. The accuracy of different methods for quantifying water stress tolerance was evaluated by measuring the rates of surviving and death in plants and leaves, and newly grown leaves after rewatering. In another experiment, the same genotypes were evaluated by applying 18% (w/v) of polyethylene glycol (PEG) to germinating seeds grown in paper rolls to induce osmotic stress, using relative root and shoot lengths as a measure of tolerance. The results suggested that transpiration measurements at the recovery stage could be the most sensitive method for separating contrasting genotypes. However, the method is time-consuming and laborious for large scale screening. Chlorophyll content, dry biomass, shoot water content and stomatal density did not correlate with the plant drought tolerance. At the same time, chlorophyll fluorescence F_v/F_m ratio showed a strong correlation with the drought tolerance and could be recommended as suitable proxy for screening. Measuring relative root growth rate (length) using PEG-treated paper roll-grown seedlings also seems to be a highly suitable and promising method for screening a large number of genotypes in breeding programs.

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3.1 Introduction

Plants face a multitude of hostile environmental conditions such as drought, salinity, waterlogging, extreme high and low temperatures, UV radiations, heavy metal toxicity, and nutrient deficiencies termed collectively as abiotic stresses. Among all environmental stresses drought is arguably the most severe stress affecting crop production globally. It has been estimated that climate change along with warming temperatures will create increasingly severe and prolonged drought episodes in the next 30–90 years that will affect over a third of the earth including world's best food production areas as a result of both decreased precipitation, increased evaporation or both (Cook et al., 2014; Dai, 2011; Dai, 2013). This is expected to result in significant (over 75%) losses in agricultural production worldwide costing approximately $23.5 billion per year and posing a major risk to the food security (FAO, 2015). At the same time, global food production will need to increase by up to 70% to feed over 9.3 billion people by 2050 (FAO, 2011). This may only be achieved through development of crop plants with higher drought tolerance and better adaptation to aridity-prone environment, as well as creating plants with increased water use efficiency.

Plants show a plethora of morphological, physiological and biochemical responses to drought stress and use different adaptive mechanisms. Drought stress severely reduces seed germination, plant growth and development because of the reduction of turgor pressure and cell elongation and expansion (Abdel-Ghani et al., 2015). Plants adapt to drought stress by inducing various morphological responses such as escaping dehydration by completing their lifecycle before soil dehydration, reducing transpiration by closing stomata, developing large and prolific root system, decreasing leaf area, and leaf rolling (Farooq et al., 2009). Production of compatible solutes acting both in osmotic adjustment and as osmoprotectants and antioxidant compounds are considered to be among the most important physiological and biochemical mechanisms for coping with water deficit conditions (Ashraf et al., 2011; Farooq et al., 2009). Importantly, the optimal strategies for dealing with drought stress differ significantly between species. Moreover, even within the same species such strategies may differ, depending on the severity of drought stress, stress duration, and growth and developmental

stages of plants (Gonzalez et al., 2010; Szira et al., 2008). Sensitivity to drought is determined by a decrease in many important physiological and morphological parameters such as photosynthetic rate, stomatal conductance, biomass and ultimately yield. The above mentioned plethoras of adaptive mechanisms provides a certain degree of flexibility for plants to adapt to harsh environmental conditions, and are also the traits which should be targeted in breeding programs.

Barley (*Hordeum vulgare* L.) is one of the major cereal crops used as a staple food in Europe, the Middle East, North and South Asia and Africa, America, Canada and Russia, where barley crops face seasonal or permanent water deficits during their lifecycle that affect growth and yield. Variation in physiological traits including relative water content, biomass, water use efficiency, net photosynthesis, quantum yield of PSII (F_v/F_m), chlorophyll content and stomatal conductance are mostly associated with the barley's response to drought stress (de Mezer et al., 2014; Ghotbi-Ravandi et al., 2014). Under drought conditions, net photosynthesis is reduced significantly as a consequence of reduced stomatal conductance, which has a direct effect on barley growth and yield (Gonzalez et al., 2010). The yield responses of most of the cereal crops including barley depend on the severity, duration and time of the stress, and the response after rewatering. However, barley has the capacity to produce higher yield in water deficit environments than wheat (*Triticum aestivum* L.), triticale (× *Triticosecale* sp.), and oat (*Avena sativa* L.) most likely because its extensive root system and its faster and more vigorous growth during vegetative development (López-Castañeda and Richards, 1994; Streda et al., 2011). Variation in effective root systems is therefore likely to relate directly to drought stress tolerance and yield. The impact of the drought stress seems to differ depending on the plants' phenological stage (Farooq et al., 2014; Nezhadahmadi et al., 2013), being more severe at reproductive stages (Atteya, 2003).

In addition to the physiological complexity of the drought tolerance trait *per se*, one of the reasons for the lack of a major progress in plant breeding for drought tolerance is the lack of convenient and reliable phenotyping methods that allow us to standardise the condition and screening of a large number of barley accessions in a reliable and reproducible manner (Gaudin et al., 2013; Munns et al., 2010).

Identification of key traits of drought-tolerant germplasm and developing better stress-adapted genotypes requires robust, reproducible, simple, and rapid screening protocols for efficient phenotyping, regardless of whether this is done in the field, or in laboratory, pot or hydroponic trials. Very many methods have been used in drought studies, each having its advantages and disadvantages (Gaudin et al., 2013; Szira et al., 2008).

A popular way to phenotype drought tolerance germplasm is to use pot experiments. Although these experiments lack the complex interactions of field trials, they allow much greater targeting of physiological traits and responses. Under controlled conditions, drought may be imposed by progressive withholding of irrigation, drying down the soil to a certain moisture content and then keeping it at desirable level by gravimetric methods (de Mezer et al., 2014; Earl, 2003). As the depth of the rooting media in the pots is not so high, the bottom of the soil easily become saturated during draining of the water (Passioura, 2006), so pots should be tall to enhance drainage. The limited pot size may also constrain root growth. However, the major drawback with this approach is that this method is very time-consuming and labour-intensive, which makes it unsuitable for screening a large number of genotypes. The convenience of the method is the accuracy to which the drought level is controlled and a possibility of measuring various physiological parameters at different soil moisture contents.

The next critical question is what agronomical and physiological characteristics are most suitable to use as a proxy for drought tolerance. Plant grain yield will be the ultimate test but requires the screening trail to last through the entire plant ontogeny, a time-consuming and labour-intensive approach. Therefore, different physiological (transpiration rate, stomata conductance, chlorophyll content, F_v/F_m, relative water content) and agronomical (biomass) traits are often used as suitable proxies.

Leaf transpiration, stomatal conductance or both are the most direct measures of plant water consumption. However, as the drought stress progresses stomata close, and leaves are often rolled, making these measurements unfeasible in practical terms. SPAD chlorophyll meter is often being used for rapid and cost-effective assessment of drought tolerance (Arunyanark et al., 2008; Filek et al., 2015;

Sharma et al., 2015). The results, however may be somewhat misleading, as although drought stress negatively affects chlorophyll biosynthesis, chlorophyll density per unit area may increase as a result of reduced leaf growth and thicker leaves in stressed plant (Rao and Wright, 1994). Another widely used proxy is the chlorophyll fluorescence and, specifically, the maximum quantum efficiency of light harvesting in PSII in dark adapted leaves (the so-called F_v/F_m ratio) (Baker and Rosenqvist, 2004). Being very rapid and noninvasive, the chlorophyll fluorescence measuring technique is considered to be an effective, reliable, and reproducible diagnostic tool for high-throughput assessments of plant germplasm for drought tolerance (Arunyanark et al., 2008; Baker, 2008; Sharma et al., 2015). Other chlorophyll fluorescence characteristics such as PSII operating efficiency (F_q'/F_m'), PSII maximum efficiency (F_v'/F_m') and the PSII efficiency factor (F_q'/F_v') may be also used (Baker, 2008; Beneragama et al., 2014; Ghotbi-Ravandi et al., 2014; Oukarroum et al., 2009).

The aim of this study was to develop a reliable and reproducible way of imposing of the drought stress on plants, and to assess the suitability of various physiological and agronomical indices as proxies for barley drought tolerance for high-throughput screening of barley germplasm.

3.2 Materials and Methods

Seeds of six barley genotypes (*Hordeum vulgare* L. cvv Gairdner, Franklin, Fleet, Commander, Clipper and ZUG293) were obtained from the Australian Winter Cereal Collection and multiplied in the field at Tasmanian Institute of Agriculture (TIA) facilities in Launceston. These genotypes were selected based on their growth habitat. Gairdner and Franklin are commercial varieties grown in high rainfall areas in Australia. Fleet and Commander are typically used in relatively low rainfall areas, and the Chinese variety ZUG293 is used in drought-prone areas in China. Seeds were surface sterilized with 10% commercial bleach (NaClO 42 g L^{-1}; Pental Products, Shepparton, Australia) and thoroughly rinsed with tap water. Seeds were sown at 10 mm depth in 2 L pots using the same amount of standard potting mixture with slow-release mixed fertilizers in each pot (Adem et al., 2014). After germination, barley seedlings were thinned to seven uniform and healthy plants in each pot. Plants were irrigated with a tap water and grown under

controlled glasshouse conditions (day length, 14 h; day: night temperatures, 25°C: 15°C; relative humidity, 65%) at the University of Tasmania, Hobart, Australia. Pots were wrapped with plastic bags to prevent soil evaporation. Drought was imposed on 20-day-old seedlings (tillering stage) by withholding irrigation, as plants at this stage were previously shown to be the most sensitive to drought (Samarah, 2005; Basnayake et al., 2006), with reported grain yield reductions being as high as 50% in barley. Plants were gradually brought to 10% soil water content (see below) and kept at that level by maintaining the constant pot weight on a daily basis for two weeks. The experiment was conducted as a complete random design (CRD), with six replications for each cultivar for each of drought and control treatments. Control plants were grown at normal irrigated conditions and watered twice daily.

3.2.1 Determining the soil water holding capacity

For determining the soil water holding capacity, five uniform-sized pots were filled with potting mix and then watered to excess for saturation. Pots were allowed to drain overnight and then weighed to obtain the pot's wet weight (W_1). The pots were then allowed to dry in an oven at 60°C until they reached a constant weight, which was termed as the pot's dry weight (W_2). Soil water content (W_S) was determined by subtracting the post dry weight from wet weight ($W_S = W_1 - W_2$). Dry soil weight (W_D) was determined by deducting the weight of the empty pot weight (W_P) from the pot dry weight ($W_D = W_2 - W_P$). The target soil water content (W_T) was determined from the relative soil water content (% RSWC) as: $W_T = W_P + W_D + \%RSWC \times W_S$

3.2.2 Transpiration rate

To determine the transpiration rate, pots were covered with a plastic bag that was tied securely around the base of the plant's stem with cable ties (Fig. 3.1b). Pots were weighed on a digital balance at around noon (12.00 hours) each day. Transpiration rates were calculated by measuring the loss of water per plant during 1-h interval. Measurements were taken daily. Three replications for each cultivar were measured for each of drought and control treatments.

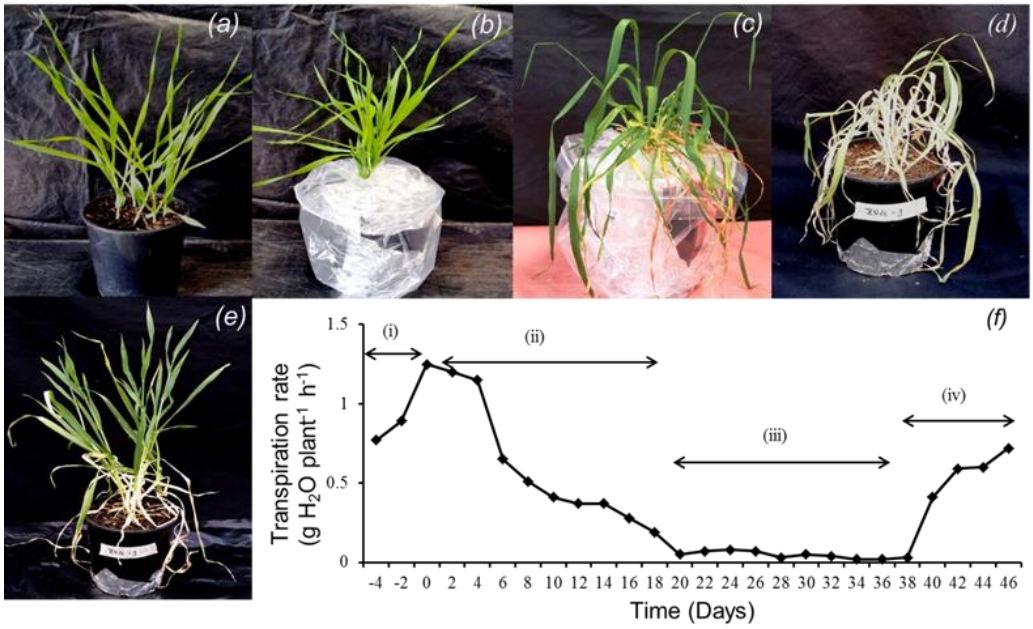


Fig. 3.1 Barley plant transpiration rate during progressive drought and recovery. (a-e) Plant phenotypes at different stages of the experiment: (a) plants at the four-leaf stage immediately before withholding irrigation; (b) pots were covered with a plastic bag to prevent evaporation from the pot before imposing drought; (c) plants at the wilting stage; (d) plants at the end of the drought period; (e) the same plants 1 week after rewatering. (f) Kinetics of the transpiration rate measured in one barley genotype (cv. ZUG 293) pictured above. One (of seven) typical examples is shown. Lower case letters shown in brackets in (f) depict different periods of the stress response: (i) well-watered plants; (ii) progressive drought until the soil moisture level reached a constant 10%; (iii) plants kept under severe (10% soil moisture) conditions for 2 weeks and (iv) rewatering stage.

3.2.3 Chlorophyll fluorescence characteristics

The maximal photochemical efficiency of PSII was estimated by measuring the chlorophyll fluorescence F_v/F_m ratio from dark-adapted samples using Optiscan OS-30P fluorometer (Opti-Science). Measurements were conducted on upper surface of the second uppermost leaves from control and drought-stressed plant at night.

3.2.4 SPAD measurements

Leaf chlorophyll content was quantified as a SPAD index with the Minolta SPAD-502 (Konica Minolta Sensing). Measurements were conducted from the middle of the lamina of the second uppermost leaves.

3.2.5 Dry biomass and water content

To determine dry biomass and water content, plants were weighed to obtain the FW immediately after harvesting and then dried for 72 h at 60°C in a drying oven and weighed using a digital balance. Water content (*WC*) was calculated as:

$$WC = \frac{FW - DW}{DW}$$

3.2.6 Recovery from the drought

The water stress tolerance of the six barley genotypes was determined by measuring the survival of seedlings after rewatering the pots back to their full water holding capacity. Two days after rewatering, the numbers of surviving and dead plants per pot, and the number of dry and living leaves per plant were measured. The number of newly grown leaves per plant, including the position of leaves, was counted after 1 week of rewatering.

3.2.7 Drought stress tolerance index

The numbers of newly grown leaves during the recovery period were counted for each pot and divided by the total number of plants in a pot (seven plants), and the average values of three replications were used as a quantitative estimate of drought tolerance, termed drought tolerance index. These drought tolerant indices were used as a standard comparison to test the effectiveness of different techniques for screening plant water stress tolerance.

3.2.8 Stomatal density

Stomatal density in barley leaves was quantified by making leaf imprints. A thin layer of nail polish was added onto abaxial surface of the middle portion of second uppermost fully expanded leaves. Once dried, the imprints were peeled off with fine forceps and placed onto a microscope slide and covered with a coverslip.

Imprints were examined microscopically at 100× magnification. The number of stomata was counted from each field of view and stomatal density (number of stomata per unit of surface area) was calculated. The sample size for each genotype was 60 (five fields of view × two imprints × six biological replications).

3.2.9 Seedling test in polyethylene glycol media

To assess the roots' contribution to overall drought tolerance, a screening assay that quantified the relative rate of root growth in a medium containing various concentrations of polyethylene glycol (PEG), a chemically inert osmolyte that reduces a solution's osmotic potential and mimics drought stress, was conducted via a two-step method. The aim of first experiment was to determine the optimal PEG and treatment duration required to reach a 50% reduction of root length and germination. This experiment was carried out at five different PEG concentrations: 10%, 15%, 18%, 20% and 25% (w/v) PEG 6000 on two genotypes (Gairdner and ZUG293). These two varieties were selected for the PEG experiment because of their contrasting drought tolerance (Gairdner, sensitive; ZUG293, tolerant) based on our preliminary drought survival screening. Once the optimal PEG concentration was chosen, the second experiment was conducted, which included all six genotypes grown under a 18% (w/v) PEG 6000 treatment (resulting in an osmotic potential of -1.14 MPa) that caused ~50% growth inhibition. Fifteen seeds were germinated on two layers of paper towels in plastic pots at 25°C under dark conditions in a growth chamber. The control plants were wetted with distilled water and the stressed plants treated with PEG 6000 solutions. After 6 days, the root and shoot length of the germinated seed were recorded. For each of the genotypes/treatment, 10 seedlings from three biological replicates were analysed.

3.2.10 Statistical analysis

All data were analysed by using SPSS ver. 20.0 for Windows (SPSS Inc.). Significant differences between different genotypes were determined by one-way ANOVA based on Duncan's multiple range test. The significance of correlations between different parameters was determined by bivariate correlations based on Pearson's correlation (two-tailed).

3.3 Results

3.3.1 Analysis of transpiration changes in response to drought stress

After imposing drought stress, transpiration rate declined gradually (Fig. 3.1f). After 1 week of withholding irrigation, plants started to wilt and transpiration rate declined to ~48% of the initial level. A similar decreasing trend was found for all six varieties (Fig. 3.2). The transpiration rate was more or less similar for all genotypes before imposing the stress. After 1 week, plants started to wilt and a significant difference in the transpiration rate was observed among the genotypes (Fig. 3.2a; $P < 0.05$). Commander was the most efficient in reducing transpiration, whereas Clipper had the highest relative transpiration rate (Fig. 3.2a, insert). The differences between other genotypes were not statistically different (at $P > 0.05$). After being stressed for 3 weeks and reaching 10% water holding capacity, the transpiration rate was very low in all genotypes (0.06 ± 0.02 g H_2O per plant per h), which represented a decline by 94-98% compared with the initial values. After rewatering, the transpiration rate increased gradually but reached only 40-45% of the initial transpiration rate (Fig. 3.1f; Fig. 3.2b). A large difference was observed in the rate of transpiration among all genotypes after re-watering. Genotypes ZUG293, Commander and Clipper transpired twice as much as the other three genotypes (Fig. 3.2b).

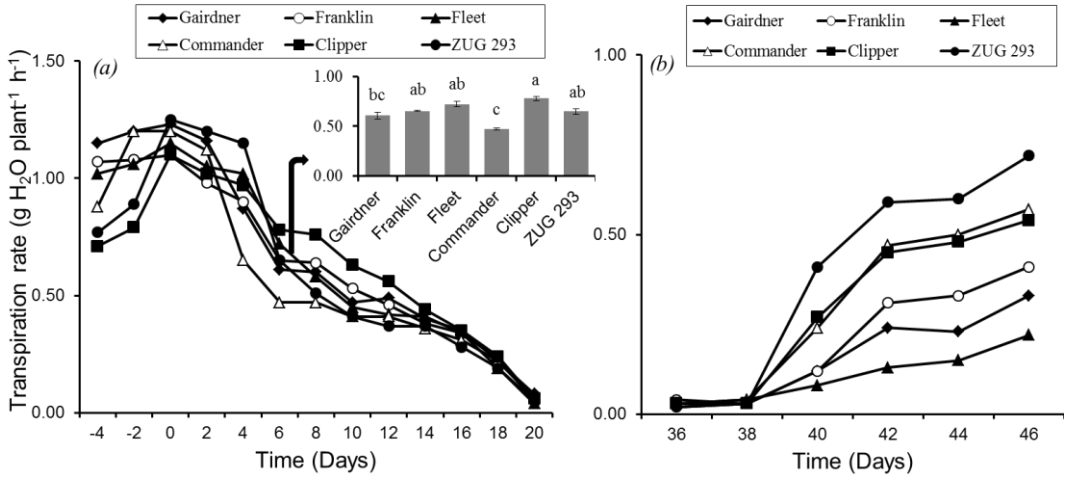


Fig. 3.2 (a) Transpiration rate of barley plants during progressive drought. Data are means ± SE, n = 21 (three pots × seven plants each). The insert in (a) shows the transpiration rate at wilting stage. Data labelled with different lower case letters are

significantly different at $P < 0.05$. (b) Genotypic differences in the transpiration rate after rewatering.

3.3.2 Survival of barley genotypes under severe drought conditions

Different survival rates were observed among the six barley genotypes after rewatering when evaluated on the basis of the number of surviving and dead plants, the number of live and dead leaves, and the number of newly grown leaves (Table 3.1). The highest resistance to severe drought was seen in genotype ZUG293. The next most tolerant genotype was Clipper, with six out seven plants surviving in each pot (Table 3.1). Low resistance to drought was observed in the genotypes Gairdner and Fleet, for which four and three seedlings died in each pot, respectively. The genotypes Franklin and Commander had intermediate drought tolerance, having five seedlings surviving in each pot. In all genotypes, up to five leaves usually died. Fully recovery was not observed in all seedlings in all genotypes. In the genotypes Gairdner and Franklin, growth was inhibited to the eighth leaves stage following 1 week of recovery. On the other hand, Clipper and ZUG293 developed ninth and tenth leaves, respectively, supporting the notion of their higher drought tolerance and adaptive capability to adapt to severe drought. The number of newly grown leaves per plant in each pot after 1 week of rehydration (Table 3.1) was determined. Genotype ZUG293 produced the highest number of (3.29) new leaves per plant followed by Clipper (2.29) and Commander (1.86). In contrast, Gairdner, Fleet and Franklin developed 0.57, 0.86 and 1 new leaves per plant, respectively. This result also indicated drought tolerance and capability to survive the imposed prolonged drought after 1 week of recovery. The drought tolerance index was estimated from the number of newly grown leaves per plant. Based on this index, barley genotypes were clustered into four groups according to their survival ability under severe drought conditions: highly tolerant (ZUG293; tolerance index 3.29), tolerant (Clipper and Commander, 2.29 and 1.86, respectively), sensitive (Franklin and Fleet, 1 and 0.86, respectively); and highly sensitive (Gairdner, 0.57) (Table 3.1).

Table 3.1 Survival ability of six barley genotypes under severe drought (10% soil water content) conditions given by the number of surviving plants and dead plants, and the number of living leaves 2 days after re-watering and the number of newly grown leaves 1 week after rehydration.

Genotype	Surviving plants per pot (n)	Dead plants per pot (n)	Living leaves per plant (n)	Dead leaves per plant (n)	Newly grown leaves per plant (n)	Newly grown leaf position (plants per pot)				Tolerance index categories
						7	8	9	10	
Gairdner	3	4	0.29	5.71	0.57	2	2	0	0	Highly sensitive
Fleet	4	3	0.43	5.57	0.86	4	2	0	0	Sensitive
Franklin	5	2	0.71	5.43	1.00	5	2	0	0	Moderately sensitive
Commander	5	2	0.71	5.29	1.86	5	4	4	0	Intermediate tolerant
Clipper	6	1	0.86	5.14	2.29	6	5	5	0	Tolerant
ZUG293	7	0	1.00	7.00	3.29	7	7	7	2	Highly tolerant

3.3.3 Analysis of physiological and agronomical changes in response to drought

The impact of drought stress on plant performance was further evaluated by analysis of the changes in the maximum quantum yield of PSII (chlorophyll fluorescence F_v/F_m ratio), chlorophyll content (SPAD value), dry biomass and water content during drought stress. F_v/F_m values ranged from 0.80 to 0.81 among the genotypes under irrigated conditions (Fig. 3.3a). A significant variation in the F_v/F_m values, however, was found in the drought-stressed plants ($P < 0.01$; Fig. 3.3b). The highest F_v/F_m was found in ZUG293 (0.61 ± 0.08) and the lowest in Franklin (0.22 ± 0.08) and Gairdner (0.12 ± 0.06), which reduced by 20%, 58% and 68% compare to the irrigated plant, respectively. The relative F_v/F_m values of drought-stressed plants (% of control) were also significantly ($P < 0.01$) different among genotypes and ranged between 80% for ZUG293 to as low as 11% for Gairdner (i.e. a sevenfold difference). Both the absolute (Fig. 3.3b) and relative (Fig. 3.3c) values of F_v/F_m declined in the sequence ZUG293 > Clipper = Commander = Fleet > Franklin = Gairdner. A strong positive correlation (R^2 = 0.92; significant at $P < 0.01$) was seen between relative F_v/F_m values in drought-stressed plants and the drought tolerance index (estimated as several newly grown leaves; trend line in Fig. 3.3d). SPAD values ranged between 32.35 ± 1.00 to 40.64 ± 0.22 in irrigated plant (Fig. 3.4a) and increased in all genotypes under stress conditions (Fig. 3.4b). The relative changes (% of control) differed among varieties (Fig. 3.4c) and ranged between 101% for Clipper to 118% for Fleet but showed no significant ($P > 0.05$) correlation with tolerance (Fig. 3.4d).

Plant biomass (shoot DW) ranged between 1.15 ± 0.05 g and 1.61 ± 0.06 g among the genotypes under control conditions (Fig. 3.5a) and decreased in all genotypes to 0.47 to 0.56 g range under stress conditions (Fig. 3.5b). The relative values of the biomass of drought-stressed plants (% of control) were also significantly ($P < 0.05$) different among genotypes and ranged between 43% for ZUG293 to as low as 33% for Commander but was not significantly (R^2 = 0.56; $P > 0.05$) correlated with the drought tolerance index (Fig. 3.5d).

There was no significant ($P > 0.05$) difference in shoot water content among the varieties under control conditions (Fig. 3.6a), although some differences appeared

under drought stress (Fig. 3.6b). On average, the relative water content in drought-stressed plants ranged between 20% and 30% of the control, depending on the variety (Fig. 3.6c) but showed no significant ($P > 0.05$) correlation with the drought tolerance index (Fig. 3.6d).

A significant ($P < 0.01$) variation was seen in the stomatal density among the genotypes under normal irrigated conditions (Fig. 3.7a). The highest stomatal density was found in the sensitive genotypes Franklin (340 ± 8 mm^{-2}) and Gairdner (283 ± 9 mm^{-2}). The lowest stomatal density was observed in the tolerant genotypes ZUG293 (229 ± 4 mm^{-2}). No significant correlation ($R^2 = 0.32$; $P > 0.05$) was found between stomatal density and drought tolerance index (Fig. 3.7b).

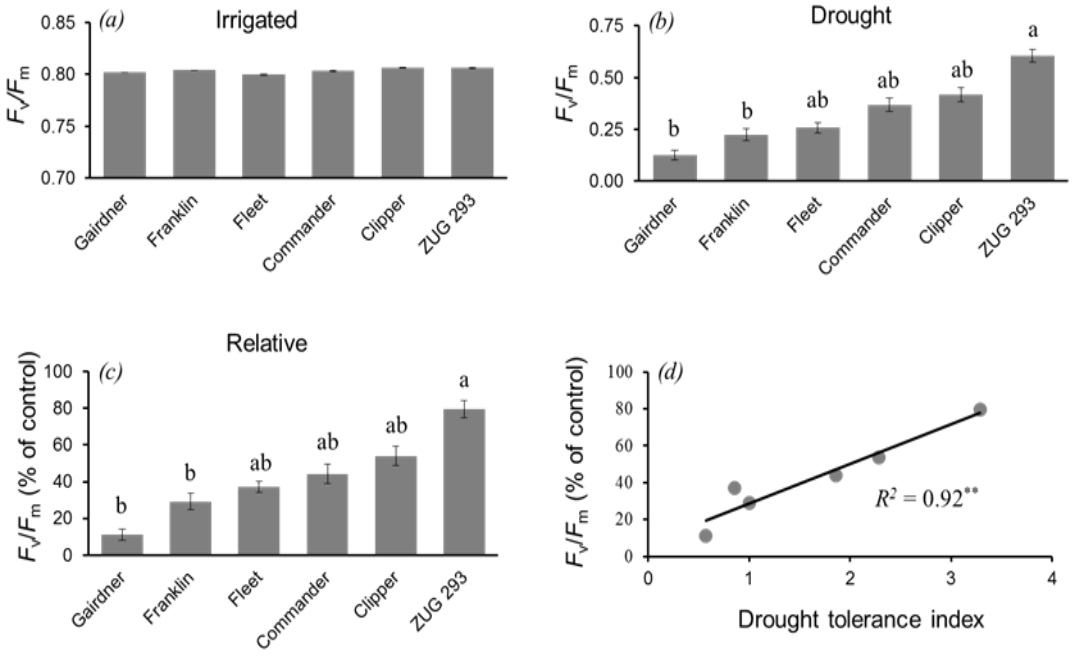


Fig. 3.3 Chlorophyll fluorescence F_v/F_m ratio in six barley genotypes under (a) control conditions and (b) after 2 week of keeping plants at 10% soil moisture level. Mean ± SE, $n = 6$. (c) Relative F_v/F_m ratio in drought-affected plants (expressed as a percentage of the control). (d) Correlation (Pearson's R^2 value) between the relative F_v/F_m ratio and the drought tolerance index estimated by the number of newly grown leaves per plant 1 week after recovery from drought. Values labelled with different lower case letters and asterisks are significantly different at $P < 0.01$.

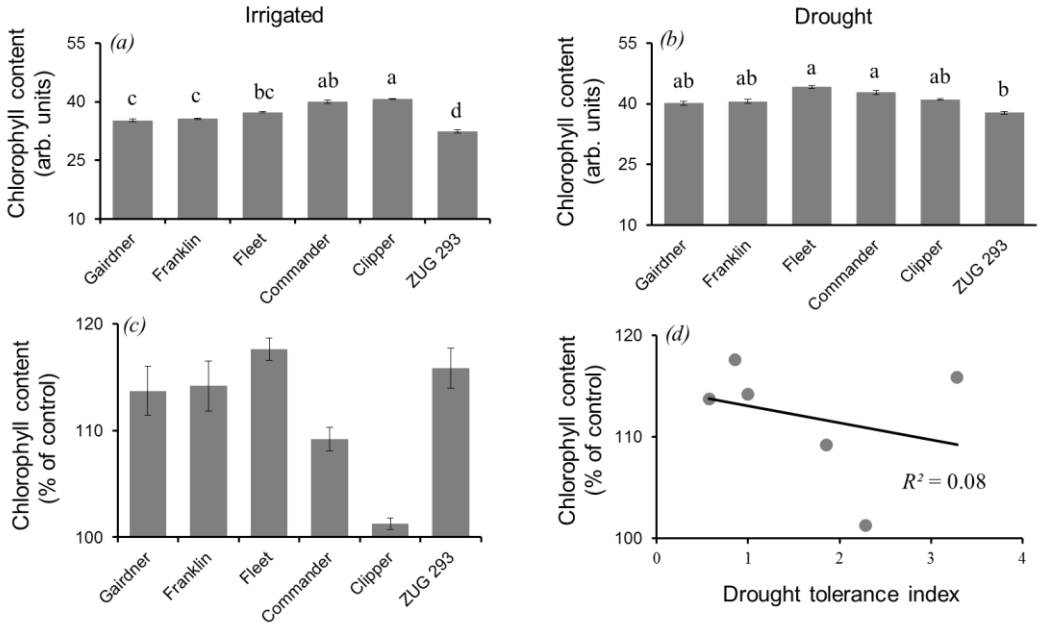


Fig. 3.4 Chlorophyll content (SPAD reading) in six barley genotypes under (a) control conditions and (b) at the end of a 2- week period of keeping plants at 10% soil moisture. Data are means ± SE, n = 6. (c) Relative chlorophyll content in drought-affected plants (expressed as a percentage of the control). (d) Correlation (Pearson's R^2 value) between the relative chlorophyll content and the drought tolerance index estimated by the number of newly grown leaves per plant a week after recovery from drought. Values labelled with different lower case letters are significantly different at $P < 0.01$.

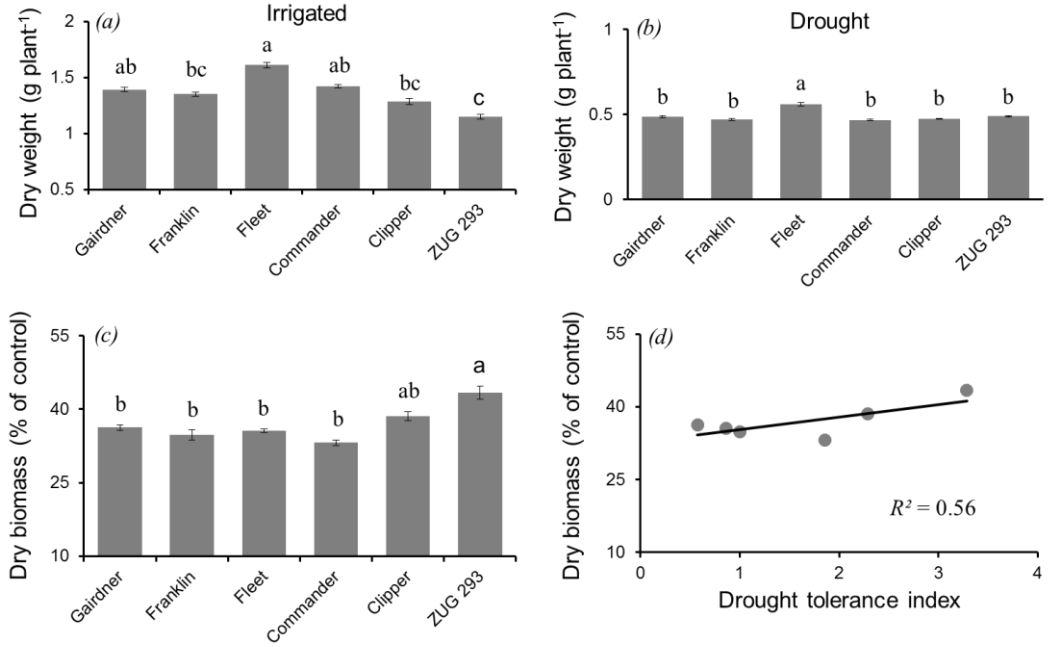


Fig. 3.5 Dry weight in six barley genotypes under (a) control conditions and (b) at the end of a 2- week period of keeping plants at 10% soil moisture. Data are means ± SE, n =

6. (c) Relative DW of drought-affected plants (expressed as a percentage of the control). (d) Correlation (Pearson's R^2 value) between the relative DW and the drought tolerance index estimated by the number of newly grown leaves per plant 1 week after recovery from drought. Values labelled with different lower case letters are significantly different at $P < 0.05$.

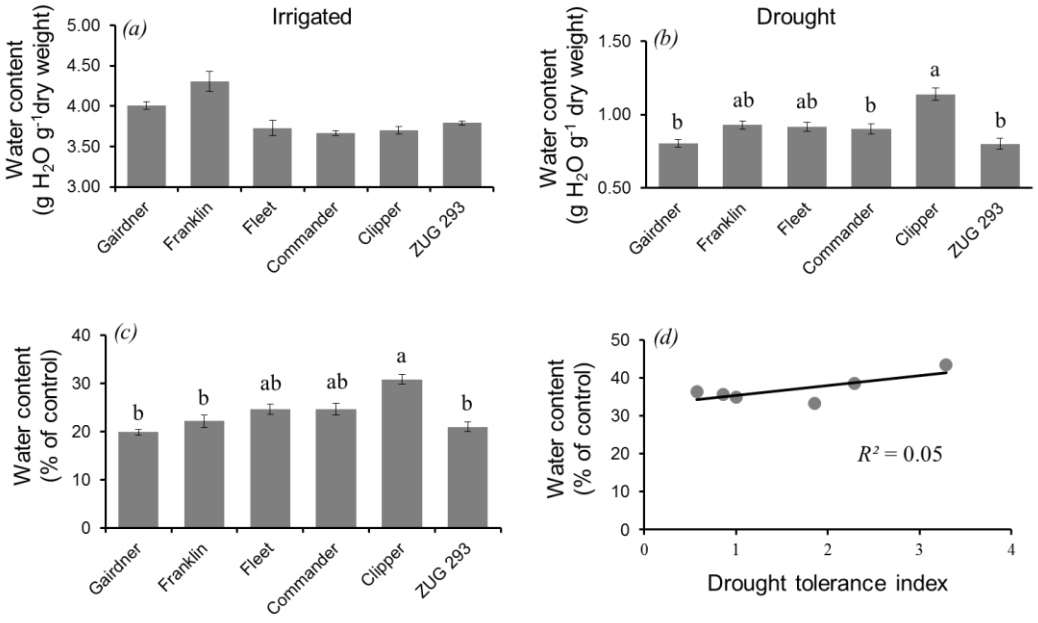


Fig. 3.6 Water content in six barley genotypes under (a) control conditions and (b) at the end of a 2-weeks period of keeping plants at 10% soil moisture. Data are means ± SE, $n = 6$. (c) Relative water content in drought-affected plants (expressed as a percentage of the control). (d) Correlation (Pearson's R^2 value) between water content (% of control) and the drought tolerance index estimated by the number of newly grown leaves per plant 1 week after recovery from drought. Values labelled with different lower case letters are significantly different at $P < 0.05$.

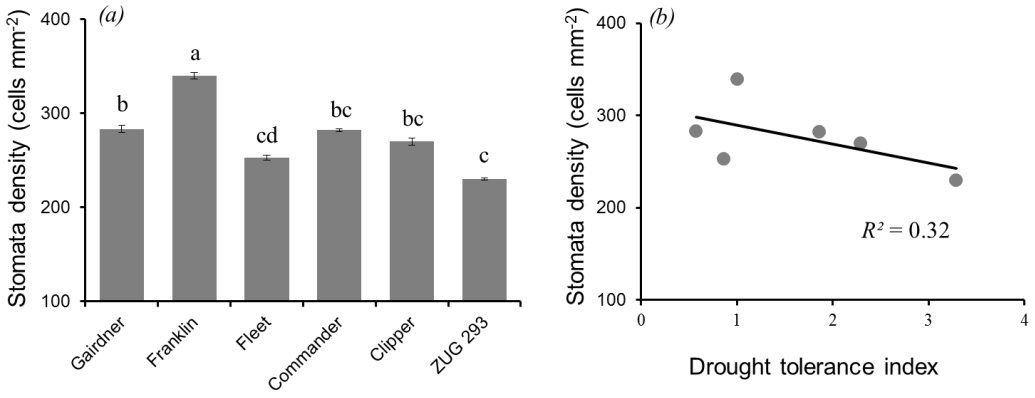


Fig. 3.7 (a) Stomatal density in six barley genotypes under irrigated conditions. Mean ± SE, n = 6. (b) Correlation (Pearson's R^2 value) between stomatal density and the drought tolerance index estimated by the number of newly grown leaves per plant one week after recovery from drought. Values labelled with different lower case letters are significantly different at $P < 0.01$.

3.3.4 Seedling test in PEG

Osmotic stress caused by PEG severely affected root growth in a dose-dependent manner (Fig. 3.8a, b). No seedling germination occurred under 25% PEG concentration in any genotype. Treatment with 18% and 20% PEG resulted in three- and fourfold reduction in root length for Gairdner, and two- and three-fold reduction for ZUG293 compared with control conditions, respectively (Fig. 3.8). Therefore, 18% PEG treatment was chosen as the most suitable for screening purposes (i.e. it resulted in ~50% growth inhibition). No significant genotypic difference ($P > 0.05$) was observed in either root or shoot growth under 18% PEG treatment among the six genotypes (Figs 3.9b and 3.10b). The highest root length was measured in ZUG293 (7.28 ± 0.25 cm), followed by Clipper (6.39 ± 0.73 cm) under osmotic stress conditions whereas highest shoot length was in Gairdner (3.60 ± 0.12 cm) and Clipper (3.37 ± 0.08 cm) (Figs. 3.9b and 3.10b, respectively). The highest relative root and shoot lengths were seen in ZUG293 (55% and 32% of the control, respectively; Figs 3.9c and 3.10c). The lowest relative root and shoot lengths were found in Gairdner (42% of the control) and Franklin (20% of the control). A significant correlation ($R^2 = 0.63$; $P < 0.05$) was found between relative root length and the drought tolerance index (Fig. 3.9d), but not between shoot length and the drought tolerance index (Fig. 3.10d).

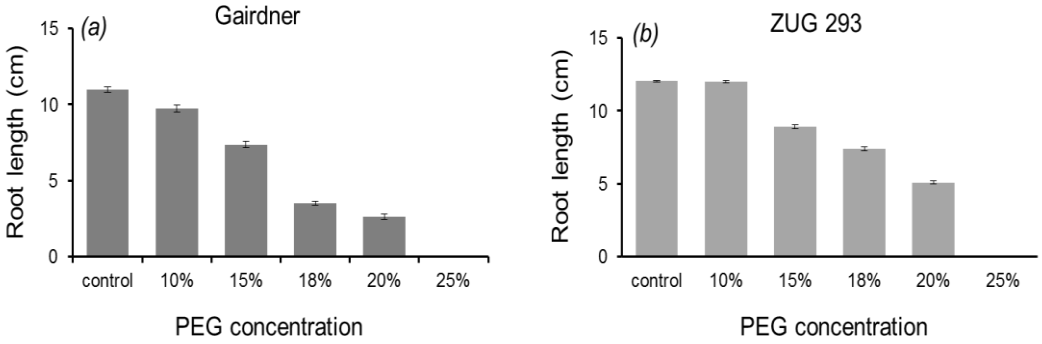


Fig. 3.8 Seedling test regarding the root length of two barley genotypes grown on two layers of paper towel for 6 days under control conditions and different concentrations of polyethylene glycol (PEG 6000) solution. (a) Gairdner (b) ZUG293. The control and stressed plants were wetted with distilled water and different concentrations of PEG 6000 solution. Data are means ± SE, n = 30 (3 replicates × 10 plants each).

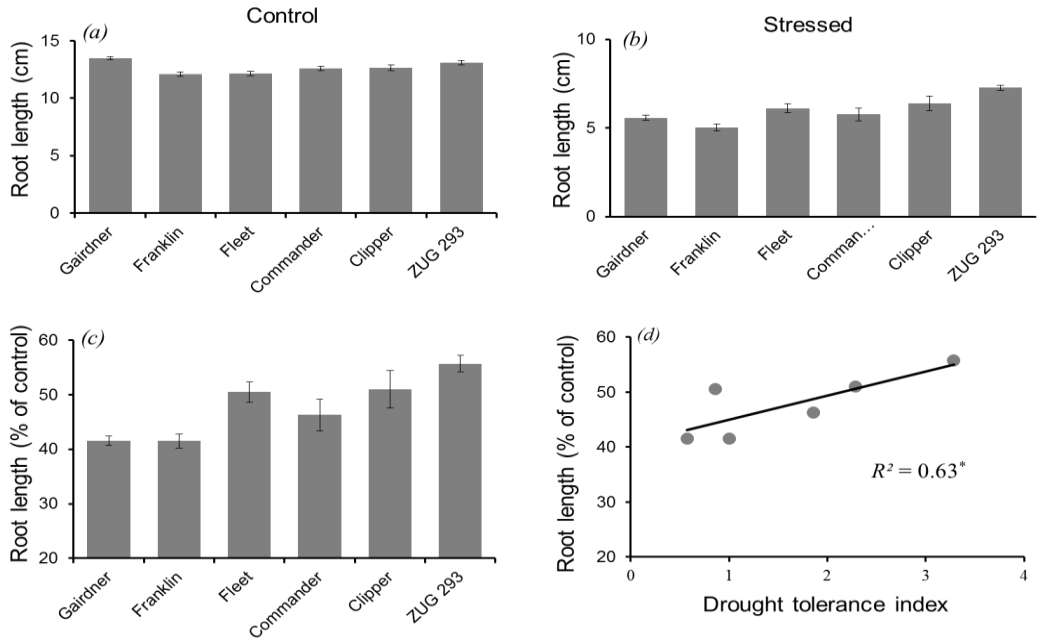


Fig. 3.9 Root lengths of six barley genotypes grown under (a) control conditions and (b) osmotic stress (18% polyethylene glycol 6000 solution). Data are means ± SE, n = 30 (3 replications × 10 plants each). (c) Relative root length in osmotically stressed roots (% of control). (d) Correlation (Pearson's R^2 value) between the relative root length and the drought tolerance index estimated by the number of newly grown leaves per plant 1 week after recovery from drought. Values are insignificant at $P > 0.05$.*, significant at $P < 0.05$.

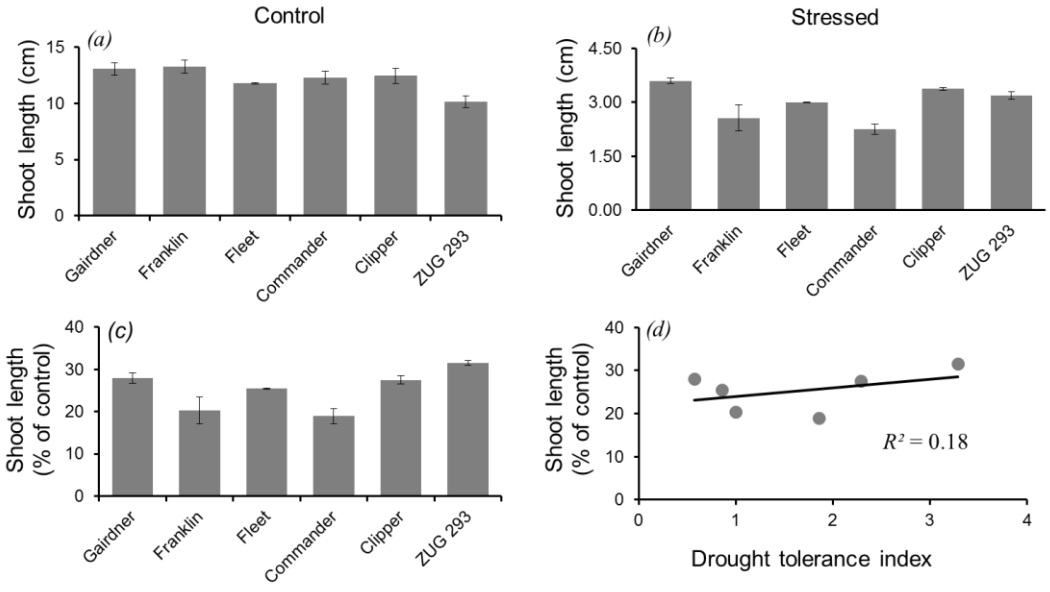


Fig. 3.10 Shoot length in six barley genotypes grown under (a) control conditions and (b) osmotic stress (18% polyethylene glycol 6000 solution. Data are means ± SE, n = 30 (3 replications × 10 plants each). (c) Relative shoots length in osmotically stressed plants (% control). (d) Correlation (Pearson's R^2 value) between the relative shoot length and the drought tolerance index estimated by the number of newly grown leaves per plant one week after recovery from drought. Values are insignificant at $P >$ 0.05.

3.4 Discussion

Several methods were adapted to induce drought stress and evaluate the agronomical and physiological characteristics in six barley genotypes that are linked with tolerance and adaptability to water deficit. As shown above, some of the methods had good predictive values, correlating with the ultimate reduction in grain yield (Suppl. Fig. 3.1) and could therefore be recommended as a proxy for drought tolerance, whereas others showed little, if any, correlation with the plants' ability to tolerate drought stress. The possible reasons for these differences are discussed below.

3.4.1 Using the response curve of leaf transpiration to evaluate drought tolerance

The first response of plants under drought stress is the closure of stomata to restrict transpirational water loss (Escalona et al., 2015; Heinemann et al., 2011;

Messina et al., 2015). Under severe drought conditions, stomata respond to chemical signals like ABA, causing stomatal closure and reduced transpiration (Ma and Qin, 2014). Thus stomata close progressively as drought progresses, resulting in a parallel decline in net photosynthesis caused by limiting CO_2 uptake by the leaves and affecting the metabolism, thereby inhibiting leaf, stem and total biomass of the sensitive genotypes. After rewatering, tolerant genotypes produced new leaves, which might help to increase the transpiration rate by opening stomata. Therefore, measuring transpiration could be a good technique for screening drought tolerant genotypes but it is very time- and labour- consuming.

3.4.2 Chlorophyll content, dry biomass, shoot water content and stomatal density do not correlate with plant drought tolerance

SPAD values increased in all genotypes under drought stress compared with control plants but no significant correlation was found between chlorophyll content and the plant drought tolerance index estimated by the number of newly grown leaves after rewatering. In general, chlorophyll content can be affected by drought stress as a result of increased accumulation of reactive oxygen species (Noctor et al., 2014; Sharma et al., 2012), which lead to lipid peroxidation and, consequently, chlorophyll destruction. However, in our study, chlorophyll content increased in all genotypes under drought conditions. One potential explanation is that the leaf area of barley plants was greatly reduced and the leaf thickness was increased (Maréchaux et al., 2015; Onoda et al., 2011; Wright et al., 2005), causing more chlorophyll per unit area of leaves under drought stress conditions. The thicker drought-affected leaves have higher chloroplast density per unit leaf area, which could be a strategy adopted by plants to resist drought stress. The ability to maintain chlorophyll density under drought conditions has been suggested as an important component of the drought resistance mechanism (Guo et al., 2008; This et al., 2000) and can be used as a measure of drought tolerance. However, in previous studies, the chlorophyll content of the plant either increases only slightly or remains unchanged under drought stress conditions in diverse group of plants (Kulshreshtha et al., 1987; Mensah et al., 2009; Nikolaeva et al., 2010). Chakraborty et al. (2015) reported an initial rise in leaf chlorophyll content under a light to moderate level of drought stress, although the chlorophyll content dropped significantly under severe water deficit stress in peanut. Thus from the

existing literature, it seems that there is less chance of getting a good correlation between leaf chlorophyll content and drought tolerance. In our study, changes in SPAD chlorophyll content values did not correlate with the drought tolerance index (Fig. 3.4) and hence were deemed to be unsuitable for screening the barley genotypes under severe drought stress.

During water stress, a large decrease in shoot biomass was observed in the genotypes that showed the highest biomass under control conditions (Fig. 3.5a-c), but no significant correlation (Fig. 3.5d) was found between changes in plant biomass and the drought tolerance index (Table 3.1). Under prolonged drought stress conditions, plants could adapt to drought by having smaller leaves, fewer tillers and reduced leaf area (Farooq et al., 2009). In addition, drought stress inhibits cell elongation and expansion (Jaleel et al., 2009), resulting in decreased plant height and growth. The reduced total leaf area resulted in a reduction in net assimilate production, even though photosynthesis per unit of area might remain unchanged. This may be the reason for reduced dry matter accumulation and biomass production in sensitive genotypes under drought stress.

Genotypes that were more drought-tolerant had more shoot water content (% of control), except for ZUG293 (Fig. 3.6c) but this parameter did not significantly correlate with the tolerance index (Fig. 3.6d). High shoot water content is ultimately related to the plant's ability to retain water and results from efficient osmotic adjustment, as well as reduced transpiration rate, less elasticity of cell tissue or both, which increased the tolerance mechanism of plants under water deficit conditions (Boyer et al., 2008). Tolerant genotypes have lower stomatal density than the sensitive under normal growth conditions (Fig. 3.7a). Stomatal density and the drought tolerance index have a nonsignificant negative relationship under control (irrigated) conditions (Fig. 3.7b). Stomata, as the main portals of gas exchange during transpiration, play a critical role in CO_2 assimilation and C fixation in photosynthesis, which ultimately contribute to increase plant biomass and yield (Hetherington and Woodward, 2003). Increased stomatal density provides the capacity for rapid increases in the stomatal conductance of a leaf, maximizing CO_2 diffusion into the leaf during favourable growth conditions, which enhances the leaf's photosynthetic capacity (Tanaka et

al., 2013). Zhu et al. (2015) showed that salt-sensitive barley genotypes contained higher stomatal density than salt tolerant and wild barley genotypes under normal growth condition. This may be important for plants in order to improve their water use efficiency. Plant increases their water use efficiency by reducing maximum stomatal conductance via reduced stomatal density (Franks et al., 2015). Generally, stress-tolerant barley genotypes have a lower yield performance because of less CO_2 assimilation and biomass for reduced stomatal density under control conditions but they have better survival capacity under hostile environmental conditions than the standard cultivated genotypes because of increased water use efficiency.

3.4.3 Chlorophyll fluorescence F_v/F_m ratio showed a strong correlation with drought tolerance and can be recommended as suitable proxy for screening

Chlorophyll fluorescence measures the efficiency of operation of PSII and has been often suggested as sensitive indicator of resistance to a broad range of environmental stresses (Kautz et al., 2014). A positive correlation between the relative chlorophyll fluorescence F_v/F_m ratio (% of control) and the drought tolerance index (Fig. 3.3d) indicates that function of PSII was inhibited and that this inhibition was more marked in sensitive genotypes. The F_v/F_m values in newly grown leaves after rewatering were close to 0.83 (a theoretical limit for F_v/F_m; (Maxwell and Johnson, 2000) indicating that tolerant genotypes were able to repair or rebuild components of PSII and the energy transfer chain after exposure to severe drought stress conditions, as described before (de Mezer et al., 2014; Ghotbi-Ravandi et al., 2014). Chlorophyll fluorescence parameters have been suggested as selection criteria for drought tolerance and for screening a large number of barley genotypes for breeding (Guo et al., 2008; Li et al., 2006), and our work here further validates their suitability. The measurements are quick and noninvasive, require a minimal expertise, and can rapidly generate large number of data for plant breeders.

3.4.4 Root but not shoot length showed a strong correlation with drought tolerance and can be recommended as suitable proxy for screening

Plant drought tolerance is determined not only by aboveground traits such as stomata conductance or density but also belowground traits such as root hydraulic

conductivity and maintenance of root growth under water stress. Since roots are the key plant organ for water and nutrient uptake from soil, root growth habits, architecture, distribution, structure, density, size and proliferations are key responses for adaptation to drought stress. Deep, thick and extensive root system are able to uptake water from a deeper layer of soil under water deficit conditions, which may be considered to be an important selection criterion for drought-tolerant genotypes. High molecular weight PEG has been used as an osmolytes to impose a controlled water deficit (Barati et al., 2015; Munns et al., 2010). PEG with a high molecular weight (6000 g mol^{-1}) is a nonpenetrating, water-soluble and nonionic polymers that induces drought stress by causing osmotic stress (George et al., 2015; Khakwani et al., 2011). In this study, the results showed that increasing PEG concentrations decreased the root and shoot length of genotypes compared with their controls (Figs. 3.9 and 3.10). The reduction of root (% of control) but not shoot length correlated with the drought tolerance index (Figs. 3.9 and 3.10d). The root length reductions under PEG-induced drought stress may be associated with reduced cellular division and elongation during germination (Fraser et al., 1990). Variation in the sensitivity of contrasting genotypes may be correlated with their osmoregulation ability under PEG-induced drought stress, which causes a strong reduction of water content that affects hydrolytic enzyme activities such as α-amylase and α-glucosidase in the drought-sensitive genotypes (Muscolo et al., 2014). Tolerant genotypes have more capability for efficient water uptake or a control system that can be correlated with the accumulation of some important compatible solutes such as free proline, total soluble carbohydrates and soluble sugars in plant root. These osmolytes can function towards not only osmotic adjustment but also detoxification of reactive oxygen and hydroxyl radical species, and to stabilise the subcellular structure and macromolecules (Hatzig et al., 2014; Marcińska et al., 2013; Mittal et al., 2015). Drought induced by osmotic stress treatment with an 18% PEG solution reduced root growth with considerable genetic variation in a large number of barley genotypes (Abdel-Ghani et al., 2015). Thus the ability to develop extensive root systems contributes to variation among genotypes for drought tolerance. Root length is considered to be an important trait for screening drought-resistant genotypes for breeders (Abdel-Ghani et al., 2015; Muscolo et al., 2014).

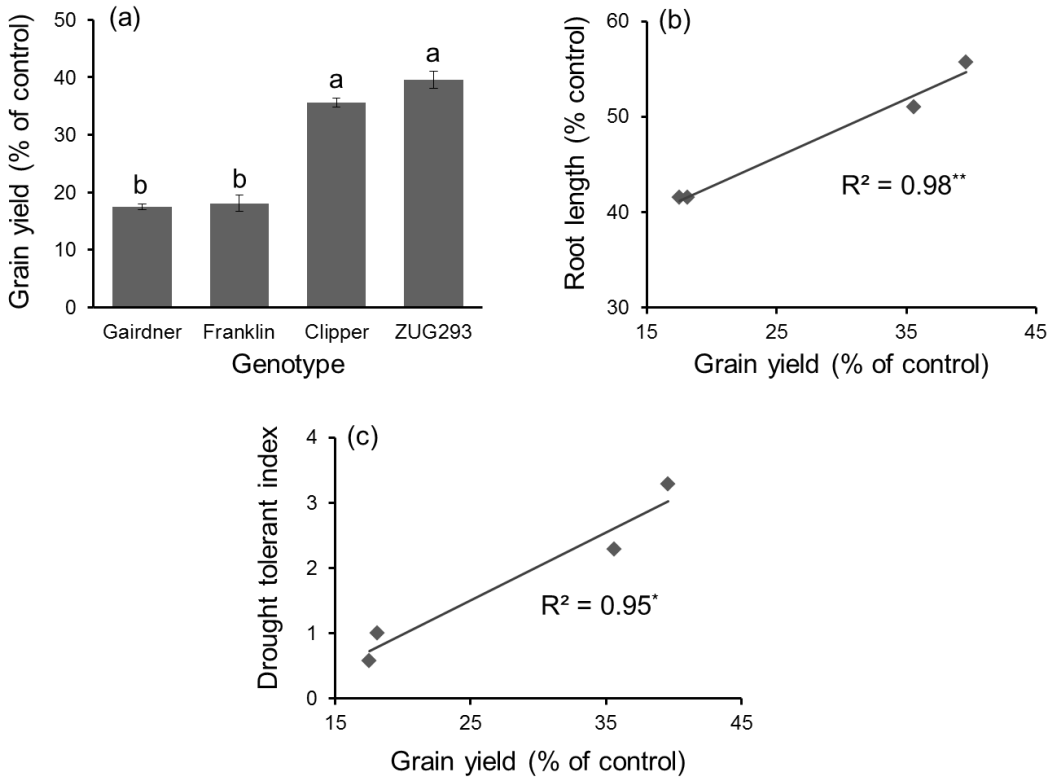
Therefore, it is suitable to compare a large number of genotypes within a short period of time by taking multiple measurements of root morphological traits in controlled environmental conditions.

3.4.5 Survival of barley genotypes after rehydration

Efficient recovery after rewatering may play an important role in plant drought adaptation. In this study, large variations in the survival characteristics were observed among the genotypes after rewatering at 7 days (Table 3.1). The best survival performance, indicating a high capacity to adapt to the drought stress conditions, was shown by the tolerant genotypes ZUG293, Clipper and Commander (Table 3.1). It has been demonstrated that tolerant genotypes of barley continued to grow when rehydrated after prolonged exposure to the severe drought stress conditions (de Mezer et al., 2014). Reduced drought-associated damage to plant photosynthetic systems is the basis of rapid recovery after rewatering. The ability of a genotype to maintain a higher chlorophyll content and F_v/F_m under drought stress conditions contributes to quick and efficient drought recovery and regrowth (Chen et al., 2015). In the present study, tolerant genotypes maintaining higher chlorophyll content and photochemical activity (F_v/F_m ratio) during prolonged drought stress could restored the photosynthesis system, thus contributing to the rapid recovery of photosynthesis and regrowth, as described by several authors (Hura et al., 2015; Rivas et al., 2016). After exposure to drought, plants increase reactive oxygen species production, including superoxide radicals, hydroxyl radicals, hydrogen peroxide and singlet oxygen; this leads to the oxidative damage and inhibition the normal cell function (Foyer and Noctor, 2005). To deal with this issue, plants have developed an elaborated antioxidant system (Apel and Hirt, 2004; Farooq et al., 2014). It has been suggested that the activation of the antioxidant defence system could be mediated by the signalling of drought stress responses that increased the ability of plant to survive and recover completely upon rehydration (Furlan et al., 2015; Zhang et al., 2015). Therefore, drought recovery by rehydration after prolonged drought stress appears to be a good indicator of drought adaptation for screening drought-tolerant genotypes.

3.5 Conclusions

Drought stress tends to a wide range of physiological responses, including reduction of photosynthesis, transpiration, shoot biomass and leaf and root growth. Breeders need to identify the key traits of drought-tolerant germplasm, and perform reproducible, simple, and rapid screening protocols for quantifying the various responses in different genotypes under drought stress conditions. The comparative evaluations of the different agronomical and physiological trait measurement and screening methods for selection and screening drought-tolerant genotypes are summarised in Table 3.2. It is concluded that the maximum quantum efficiency of light harvesting in PSII in dark-adapted leaves (the so-called F_v/F_m ratio) can be a reliable, nondestructive and simple indicator of drought-tolerant germplasm and is suitable for large-scale screening in a short period of time. Survival ability after rehydration followed by prolonged drought is also a good indication of drought-tolerant genotypes. Transpiration measurements after recovery could be a good technique for identifying the tolerant genotypes but it is time-consuming and laborious. In addition, root morphological trait measurement under PEG-induced drought stress in controlled environmental conditions correlated strongly with relative plant grain yield under drought conditions (Suppl. Fig. 3.1) and is suitable to compare a large number of genotypes within a short period of time.



Suppl. Fig. 3.1 (a) Relative grain yield per plant in drought-affected four barley genotypes (expressed as a percentage of the control). (b) Correlation (Pearson's R^2 value) between the grain yield per plant (% of control) and PEG-affected root length (% of control). (c) Correlation (Pearson's R^2 value) between the grain yield per plant (% of control) and the drought tolerance index estimated by the number of newly grown leaves per plant 1 week after recovery from drought. Values labelled with different lower case letters are significantly different at $P < 0.05$.

Table 3.2 Comparative evaluation of testing methods and traits to estimate drought tolerance in barley

Approaches	**Issues**
Seedling test by PEG induced drought stress	It is very simple, quick and cheap. A large number of genotypes can be screened in a short time. Root length may be used as a proxy for screening, but shoot length is misleading and not suitable for screening.
Seedling test by drought imposed in a glasshouse in pots	
Transpiration measurement	Transpiration is a good indicator at the recovery stage but it is too time-consuming and laborious. Not suitable for screening a large number of genotypes.
Chlorophyll fluorescence F_v/F_m ratio	F_v/F_m values are easily measurable, provide reliable tolerance information and are suitable for screening a large number of genotypes.
SPAD chlorophyll reading	SPAD chlorophyll measurement not suitable for screening genotypes.
Dry biomass	Dry biomass is a poor indicator not suitable for screening genotypes.
Water content	Water content is not suitable for screening genotypes.
Stomatal density	Stomatal density measurement is time-consuming and not suitable for screening a large number of genotypes.

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Chapter 4. Residual transpiration as a component of salinity stress tolerance mechanism: a case study for barley[3]

Abstract

While most water loss from leaf surfaces occurs via stomata, part of this loss also occurs through the leaf cuticle, even when the stomata are fully closed. This component, termed residual transpiration, dominates during the night and also becomes critical under stress conditions such as drought or salinity. Reducing residual transpiration might therefore be a potentially useful mechanism for improving plant performance when water availability is reduced (e.g. under saline or drought stress conditions). One way of reducing residual transpiration may be via increased accumulation of waxes on the surface of leaf. Residual transpiration and wax constituents may vary with leaf age and position as well as between genotypes. This study used barley genotypes contrasting in salinity stress tolerance to evaluate the contribution of residual transpiration to the overall salt tolerance, and also investigated what role cuticular waxes play in this process. Leaves of three different positions (old, intermediate and young) were used. Our results show that residual transpiration was higher in old leaves than the young flag leaves, correlated negatively with the osmolality, and was positively associated with the osmotic and leaf water potentials. Salt tolerant varieties transpired more water than the sensitive variety under normal growth conditions. Cuticular waxes on barley leaves were dominated by primary alcohols (84.7-86.9%) and also included aldehydes (8.90-10.1%), *n*-alkanes (1.31-1.77%), benzoate esters (0.44-0.52%), phytol related compounds (0.22-0.53%), fatty acid methyl esters (0.14-0.33%), β-diketones (0.07-0.23%) and alkylresorcinols (1.65-3.58%). A significant negative correlation was found between residual transpiration and total wax content, and residual transpiration correlated significantly with the amount of primary alcohols. In conclusion, both leaf osmolality and the amount of total cuticular wax are involved in controlling

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cuticular water loss from barley leaves under well irrigated conditions. A significant and negative relationship between the amount of primary alcohols and a residual transpiration implies that some cuticular wax constituents act as a water barrier on plant leaf surface and thus contribute to salinity stress tolerance. It is suggested that residual transpiration could be a fundamental mechanism by which plants optimize water use efficiency under stress conditions.

4.1 Introduction

Under optimal conditions plants lose typically 95-98% water from the leaf surface via stomatal pores in a process termed stomatal transpiration. However, under some environmental conditions, a relatively large portion of evaporated water may bypass the stomata and occur through the cuticle. Depending on the species and conditions, water loss through the cuticle can be as high as 28% of the water transpired through stomata (Boyer et al., 1997; Riederer and Schreiber, 2001). Moreover, some water can escape the leaf via stomata even when they are fully closed (Caird et al., 2007; McAdam and Brodribb, 2014). Because of this, using the term "cuticular transpiration" is not always appropriate, and this process is best described as "residual transpiration". It has been estimated that leaf cuticular water permeability varies extensively among species and ranges from 10^{-7} to 10^{-4} m s^{-1} (Burghardt and Riederer, 2008; Riederer and Schreiber, 2001). Residual transpiration is usually localized to the area surrounding stomata, where there are more and larger cuticular pores (Marschner, 1995). While stomatal conductance is a dynamic process that can be rapidly controlled by ion fluxes into/out of guard cells, residual transpiration depends almost entirely on the existing (passive) lipophilic cuticular pathway of the leaf surface, and, hence cannot rapidly be adjusted to changing conditions (Blatt, 2000; Popp et al., 2005). However, when stomata are closed under salinity or drought conditions, the balance between stomatal and non-stomatal transpiration is shifted. Under severe stress conditions, when stomata are closed and stomatal transpiration is reduced to nearly zero, the difference in residual transpiration becomes a significant factor determining water use efficiency. Thus, reducing non-stomatal (residual) transpiration is a potentially useful mechanism for improving plant performance under stress conditions. Genotypes having lower residual transpiration can conserve relatively

more water under water stress conditions, and it has therefore been suggested as a selection trait in the breeding of cereals genotypes adapted to a dry environment (Clarke et al., 1991; Petcu, 2005).

Cuticular wax is the outermost hydrophobic layer of the aerial plant tissues, and plays an important role in protecting plants against biotic and abiotic environmental stresses, and acts as a barrier to excessive non-stomatal transpiration (Yeats and Rose, 2013). The main functions of cuticular waxes include maintaining equilibrium between the transpirational water loss and root water uptake by transpiration control, defending against attack by insects and pathogens, reducing water retention on plant surfaces by controlling surface wettability, controlling loss and uptake of polar solutes, and regulating the exchange of gases and vapour (Riederer and Muller, 2008). Extraction of cuticular waxes from plant parts with organic solvent increases the cuticular water permeability indicating that the wax layer is a fundamental water transport-limiting barrier of the cuticle, especially when stomata are closed (Šantrůček et al., 2004). Some reports suggested that plants that have a thicker cuticle or a cuticle containing a larger amount of waxes are more efficient in reducing non-stomatal transpiration and thus better adapted to water stress conditions (Burghardt and Riederer, 2003), and in some species total wax loads increased by 30 to 70% under water stress conditions (Kim et al., 2007). However, the correlation between residual transpiration and the thickness of cuticle and/or amount of total cuticular waxes is still not clear-cut. Some researchers found that the total amount of cuticular waxes and cuticular thickness are negatively correlated with residual transpiration in different plants (González and Ayerbe, 2010; Jordan et al., 1984; Ni et al., 2015; Premachandra et al., 1992). However, some authors reported no correlation between residual transpiration and waxes (Ni et al., 2012; Riederer and Schreiber, 2001; Sánchez et al., 2001).

Residual transpiration could be influenced by the characteristics of the leaf surface and morphological structure of the plant. Some studies argued (Riederer and Schreiber, 2001) that residual transpiration did not relate to the amount of wax coverage and thickness of the cuticle but could be depended on physical properties, orientation of wax crystal structure and wax composition. It is not clear however if this conclusion can be extrapolated to all species. The cuticle layer is a

cutin-rich domain with embedded polysaccharides and an overlying layer that is less abundant in polysaccharides but enriched in waxes referred to as the cuticle proper (Yeats and Rose, 2013). The waxes are either deposited within the cutin matrix known as intracuticular wax or accumulate on its surface known as epicuticular wax crystals, or films. Cuticular waxes is a general term for the complex mixture of homologous series of very-long-chain fatty acids, primary *n*-alcohols, secondary *n*-alcohols, *n*-aldehydes, *n*-alkanes, *n*-alkyl esters, and cyclic organic compounds like pentacyclic triterpenoids, flavonoids, tocopherols and hydroxycinnamic acids derivatives (Jetter et al., 2008). Specific chemical compounds of the cuticle may be related to the water barrier. Higher levels of nonpolar long chain aliphatic wax compounds of cuticular wax such as hydrophobic alcohols, *n*-alkanes, and aldehydes tend to be associated with a barrier against cuticular water loss while alicyclic wax components including triterpenoids and sterol derivatives are less effective as a water barrier (Buschhaus and Jetter, 2012; Leide et al., 2011; Leide et al., 2007; Macková et al., 2013).

It is also not clear whether residual transpiration is only related to the cuticular wax on the leaf surface or it is also associated with the plant water relations. It was suggested that residual transpiration is correlated with leaf water status such as leaf water content, osmotic potential and leaf water potential (Clarke et al., 1991). Other evidence, however, shown that residual transpiration is not related to relative water content or osmotic potential (Rawson and Clarke, 1988).

The objectives of this study were to investigate the effect of residual transpiration on salinity tolerance and the relationship of residual transpiration to plant water relations, and cuticular wax load at three different leaf positions under irrigated conditions of two salt tolerant and two salt sensitive barley genotypes.

4.2 Materials and methods

4.2.1 Plant materials and growth conditions

Four barley (*Hordeum vulgare* L.) genotypes contrasting in their salt tolerance were used in this study. Cultivars Franklin and Gairdner were salt sensitive and failed to produce any grain when grown under highly saline (300 mM NaCl) conditions in the glasshouse (Chen et al, 2007), while cultivars TX9425 and ZUG

293 were salt tolerance and managed to produce ~ 30% grain yield (compared with control) under same conditions. Seeds were obtained from the Australian Winter Cereal Collection and multiplied in the field at Tasmanian Institute of Agriculture facilities in Launceston. Seeds were surface sterilized with 10% commercial bleach and thoroughly rinsed with tap water, and sown in 2 L plastic pots using standard potting mixture containing 70% composted pine bark; 20% coarse sand; 10% sphagnum peat; Limil at 1.8 kg m^{-3}, dolomite at 1.8 kg m^{-3}. The plant nutrient balance was maintained by adding the slow release Osmocote Plus™ fertilizer (at 6 kg m^{-3}), plus ferrous sulphate (at 500 g m^{-3}). Plants were grown under controlled glasshouse conditions (day length, 14 h; day/night temperatures, 25/15°C; relative humidity, 65%) at the University of Tasmania (Hobart, Australia) in January 2015. The plants were irrigated automatically twice per day.

4.2.2 Residual transpiration measurement

Two different methods were used for the determination of residual transpiration from the excised leaf under dark conditions as follows:

4.2.2.1 Method-1

Residual transpiration was determined following Clarke and McCaig (1982) with modification. Three fully expanded leaves from each genotype at three positions (old leaf, intermediate leaf and young flag leaf) were selected for sampling (Fig. 4.1a). The leaves were excised and sealed with vacuum grease on the cut end immediately. Then collected leaves were immediately transported to the laboratory. Fresh weights (W_0) were determined by an electronic balance. The leaves were then placed in a controlled dark room at 20-21°C and 50% relative humidity (RH). The leaves were weighed at 2, 4 and 6 hour (W_2, W_4 and W_6 respectively) intervals and then placed in dry oven at 60°C for 24 h and reweighed (W_d). Residual transpiration was measured per dry weight basis by using the following formula

$$\text{Residual transpiration} = \frac{(W_0 - W_2) + (W_2 - W_4) + (W_4 - W_6)}{3 \times W_d(T_2 - T_1)}$$

where T_1-T_2= time interval between two subsequent measurements (2 h).

The measured residual transpiration was then recalculated per projected leaf area basis and expressed in mg H_2O cm^{-2} h^{-1}.

4.2.2.2 Method-2

Residual transpiration was measured according to Clarke et al. (1991) with modification. Leaf sampling was the same as for Method-1. Initial weights were determined immediately after excision of leaves. The leaves were maintained in darkness for stomatal closure under ambient room conditions at 20-21°C and 50% RH. The leaves were weighed again after 24h. The leaves were dried at 60°C for 24h and then dry weight was determined. Residual water loss was determined per dry weight basis by using the following formula

$$\text{Residual transpiration} = \frac{(W_\text{i} - W_\text{d}) - (W_{24} - W_\text{d})}{W_\text{d}}$$

where W_i = Initial fresh weight; W_{24} = Fresh weight after 24 hours; W_d = Dry weight

The measured residual water loss was then recalculated per leaf area basis and expressed in mg H_2O cm^{-2}.

4.2.3 Measurement of leaf osmolality and osmotic potential

Three leaves at three leaf position e.g. old, intermediate and young flag leaves were taken from each genotype. Representative leaf samples were taken in centrifuge tubes and frozen at -20°C overnight and then squeezed to extract sap. An amount of 10µl sap was taken from each sample for measuring leaf osmolality (c) using a vapour pressure osmometer (Vapro model 5520, Wescor Inc., Logan, Utah). The osmotic potential was calculated by Van't Hoff's equation from the osmolality (mmol kg^{-1}): osmotic potential (MPa) = -c (mmol kg^{-1}) $\times 2.4789 \times 10^{-3}$ at 25°C.

4.2.4 Measurement of leaf water potential

Two leaves were excised from each genotype from three positions of the stem for leaf water potential determinations. The leaf blades were cut with a sharp blade and immediately sealed in an elliptical grass compression gland gasket. The leaf

blades were sealed in a pressure chamber (Model 615; PMS Instruments, Albany, OR, USA), and the chamber was pressurised using compressed air at a rate of 0.1 MPa s^{-1} until water first appeared at the cut surface of the leaf. The total elapsed time from when the leaf was cut from the plant to the initial pressurisation of the chamber was 5-10 s. The leaf water potential data were reported in MPa.

4.2.5 Scanning electron microscopy (SEM)

After sampling the leaves were stored at -20°C overnight and then lyophylised in a pre-cooled freeze drier (Mini-ultra cold, Dynavac, Aus, Techno lab). The dried samples (3-5 mm long) were mounted on SEM specimen stubs with double-sided carbon tape (one half with adaxial and the other with abaxial surface uppermost) and then coated with a thin film (2-3 nm) of Pt for 20 min using a sputter coater (BalTec SCD 050) in an atmosphere of argon to improve the electrically conducting properties of leaf and high resolution of images. Three replicates of coated samples were examined with a Hitachi SU-70 UHR field emission scanning electron microscope setting with 1.5 kV, 17.2 mm × 2.00 k SE (M). The imaging was performed in the Central Science Laboratory, University of Tasmania.

4.2.6 Wax extraction and analysis

Three fresh leaves at three positions of the plant from each genotype were excised and ten 0.64 cm^2 disks were sampled from each by leaf punch. The leaf segments were soaked in 5 mL of solvent (dichloromethane with *n*-docosane (C_{22} alkane, 20 mg/L) as an internal standard) for 5 min with gentle stirring (Wu et al., 2013). The extract contained waxes from both abaxial and adaxial leaf surfaces. The extracts were evaporated to dryness under a nitrogen stream for 30 min at 58 °C. The samples were redissolved in 0.5 mL dichloromethane for analysis by combined gas chromatography-mass spectrometry (GC-MS) on a Varian 3800 gas chromatograph coupled to a Bruker-300 triple quadrupole mass spectrometer. One microlitre injections in splitless mode were made with an injector temperature of 275^oC. The column was a 30 m × 0.25 mm DB5 (0.25 µm film thickness) (Agilent, Australia) and the oven temperature program was 60^oC (held for 1 min) to 220^oC at 30^oC per minute, then to 310^oC at 10^oC per minute with a final hold

time of 5 min. The carrier gas was helium at a constant flow of 3.5 mL min^{-1}. Mass spectra were collected over the range *m/z* 40 to 600 every 0.3 s. Compounds were identified through a combination of MS reference databases (NIST MS database and an in-house database of relevant compounds), and Kovats' retention indices. The individual components and total wax were expressed in terms of µg equivalents of *n*-docosane cm^{-2}. All subsequent µg cm^{-2} values are in terms of *n*-docosane equivalents in the text and figures.

4.2.7 Statistical analysis

All data were analyzed by using SPSS 20.0 for Windows (SPSS Inc.). Significant differences between different genotypes were determined by one-way analysis of variance based on Duncan's multiple range tests. Different lower case letters in the figures represent significant differences. The significance of correlations between different parameters was determined by bivariate correlations based on Pearson's correlation (two-tailed).

4.3 Results

4.3.1 Residual transpiration

As both stomatal density and amount of cuticular waxes depends on the leaf age, we hypothesised that a significant variation in residual transpiration should exist between leaves of different positions. A significant variation was seen in the different leaf positions for all varieties ($P < 0.05$; Fig. 4.1a and b). Old leaves transpired more water than the intermediate and flag leaves for all varieties using both methods. In Method-1, significant variation was observed between old leaves and intermediate leaves but not in intermediate and flag leaves in most genotypes. Old leaves of TX9425 (0.74 ± 0.04 mg H_2O cm^{-2} h^{-1}) genotype transpired the highest amount of water and Franklin transpired the lowest amount of water (0.36 ± 0.02 mg H_2O cm^{-2} h^{-1}). In Method-2, significant differences were seen between the three leaf positions in all genotypes. Old leaves of TX9425 (10.24 ± 0.53 mg H_2O cm^{-2}) transpired the highest amount of water followed by old leaves of ZUG293 (8.01 ± 0.48 mg H_2O cm^{-2}), Gairdner (6.88 ± 0.52 mg H_2O cm^{-2}) and Franklin (6.02 ± 0.28 mg H_2O cm^{-2}), respectively. Young flag leaves of TX9425 (5.73 ± 0.25 mg H_2O cm^{-2}) transpired the highest amount of water followed by

ZUG293 (3.68 ± 0.14 mg H_2O cm^{-2}), Gairdner (3.02 ± 0.17 mg H_2O cm^{-2}) and Franklin (2.86 ± 0.12 mg H_2O cm^{-2}), respectively. Salt tolerant varieties transpired more water through the cuticle than that of sensitive varieties under normal growth conditions (Fig. 4.1c). The cumulative loss of water of the three leaf positions of two tolerant genotypes (TX9425 and ZUG293) was higher than two sensitive genotypes (Gairdner and Franklin) in both methods. The two tolerant genotypes transpired 43% and 32% more water respectively than the two sensitive genotypes in the two methods under normal growth condition.

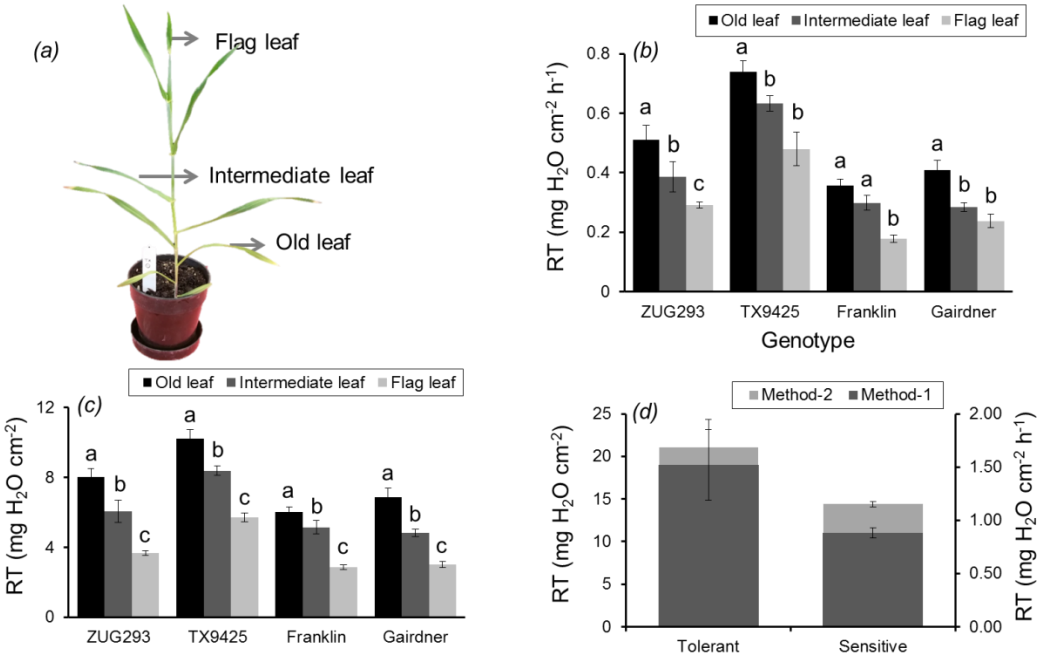


Fig. 4.1 Quantifying the residual transpiration (RT) from leaves of three different positions in barley. (a) sampled leaves; (b-c) RT values measured from leaves of three different positions from 4 barley varieties contrasting in salinity stress tolerance by Method-1 and Method-2, respectively. Data is mean ± SE, $n = 6$. (d) mean RT values for plants in salt-tolerant (ZUG293, TX9425) and salt-sensitive (Gairdner, Franklin) groups estimated by two different methods. Data labelled with different lower case letters in panels (b) and (c) are significantly different at $P < 0.05$.

4.3.2 Leaf sap osmolality correlates negatively with residual transpiration

A significant difference of leaf sap osmolality was observed among different leaf positions ($P < 0.05$; Fig. 4.2a). Leaf sap osmolality decreased with increasing leaf age for all genotypes. The osmotic potential was highest in old leaf and lowest in

flag leaf in all genotypes ($P < 0.05$; Fig. 4.3a). The highest decrease (60%) was observed in TX9425 followed by ZUG293 (43%), whereas the lowest decrease (20%) was measured in Franklin followed by Gairdner (28%), in old and young leaves respectively. A strong negative correlation (R^2 = -0.86 for Method-1 and -0.92 for Method-2; significant at $P < 0.01$) was found between the overall leaf sap osmolality in plants grown under normal growth conditions and residual transpiration.

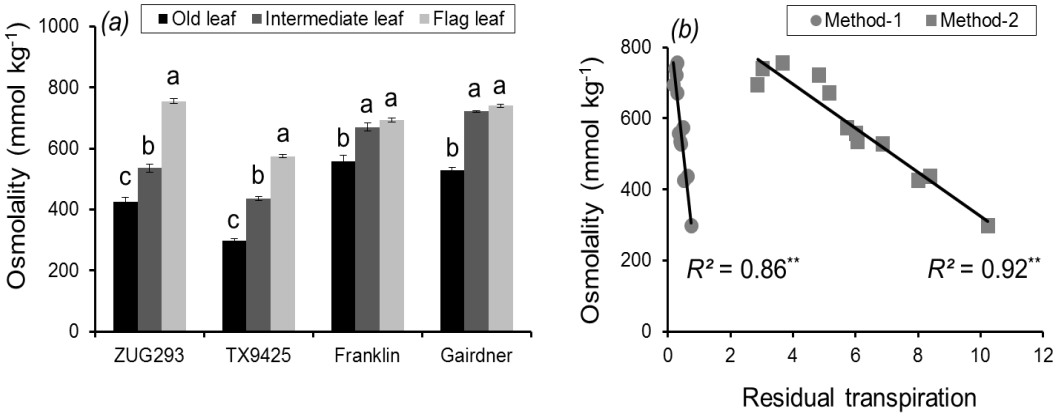


Fig. 4.2 (a) genetic variability in osmolality of barley leaves at three positions in plants grown under normal (no salt) growth conditions. Mean ± SE, $n = 6$. (b) correlations (Pearson's R^2 values) between leaf sap osmolality and residual transpiration measured by two different methods. Data labelled with different lower case letters are significantly different at $P < 0.05$ and asterisk are significant at $P < 0.01$.

4.3.3 Osmotic potential and leaf water potential correlate positively with residual transpiration

The osmotic potential was the highest in old leaves and lowest in flag leaves in all genotypes ($P < 0.05$; Fig. 4.3a). ZUG293 and TX9425 followed the order old > intermediate > young flag leaf, whereas Franklin and Gairdner followed old > intermediate = young flag leaf. A strong positive correlation (R^2 = 0.86 for Method-1 and 0.92 for Method-2; significant at $P < 0.01$) was found between the overall leaf osmotic potential in plants grown under normal growth conditions and residual transpiration. A significant variation of leaf water potential was found among the three leaf positions in all four genotypes ($P < 0.05$; Fig. 4.4a). Leaf water potential increased with increasing the plant leaf age, the highest and lowest leaf water potential was found at old leaf and young flag leaf, respectively. A

positive correlation (R^2 = 0.59; significant at P < 0.01) was found (in Method-2) between the overall leaf water potential in plants grown under normal growth condition and residual transpiration.

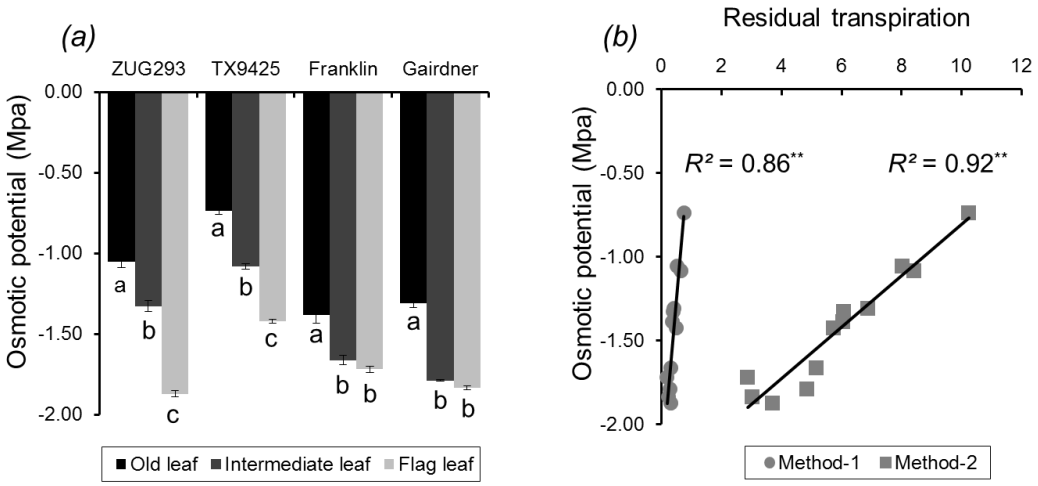


Fig. 4.3 (a) genetic variability in osmotic potential of barley leaves at three positions in plants grown under normal (no salt) conditions. Mean ± SE, n = 6. (b) correlations (Pearson's R^2 values) between leaf osmotic potential and residual transpiration measured by two different methods. Data labelled with different lower case letters are significantly different at P < 0.05 and asterisk are significant at P < 0.01.

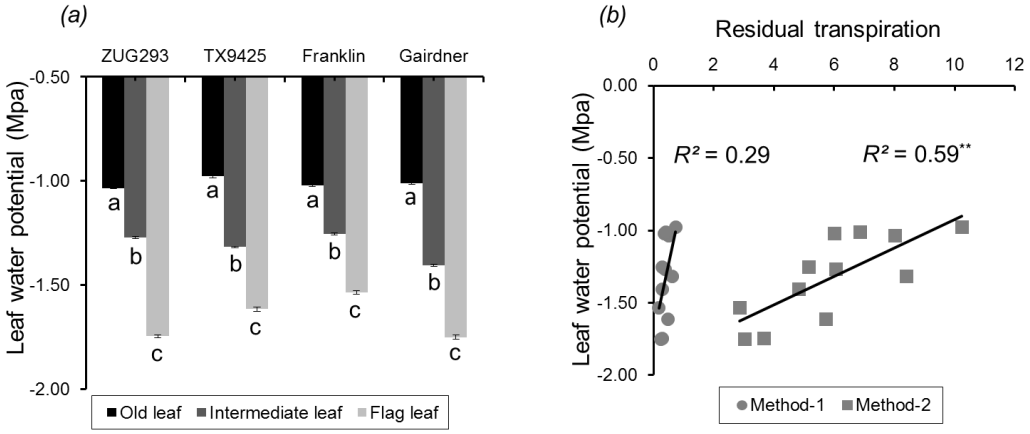


Fig. 4.4 (a) genetic variability in water potential of barley leaves at three positions in plants grown under normal (no salt) growth conditions. Mean ± SE, n = 6. (b) correlations (Pearson's R^2 values) between leaf water potential and residual transpiration measured by two different methods. Data labelled with different lower case letters are significantly different at P < 0.05 and asterisk are significant at P < 0.05.

4.3.4 Structure and distribution of cuticular waxes on leaf epidermis

SEM analysis showed similar cuticular waxes structure in three different leaf positions of four barley genotypes. The cuticular waxes formed combined coatings of different arrangement of minute crystallised plates about 1-2 µm in size, relatively vertically oriented to the leaf epidermal surface (Fig. 4.5; Suppl. Fig. 4.1). Cuticular wax structures were a less dense covering of adaxial surface of old leaves compared to the intermediate and young flag leaves for all genotypes. The epidermis of three different leaf positions of four genotypes was covered with waxy plates, but not fully over the guard cell of all genotypes (Fig. 4.6). In the case of TX9425 and ZUG293 genotypes, the guard cells of stomata were not fully covered with waxy plates, whereas the guard cell of Franklin and Gairdner were fully covered with waxy plates. No differences were found for adaxial and abaxial surface of leaves in all genotypes regarding to cuticular wax structure and density (data not shown).

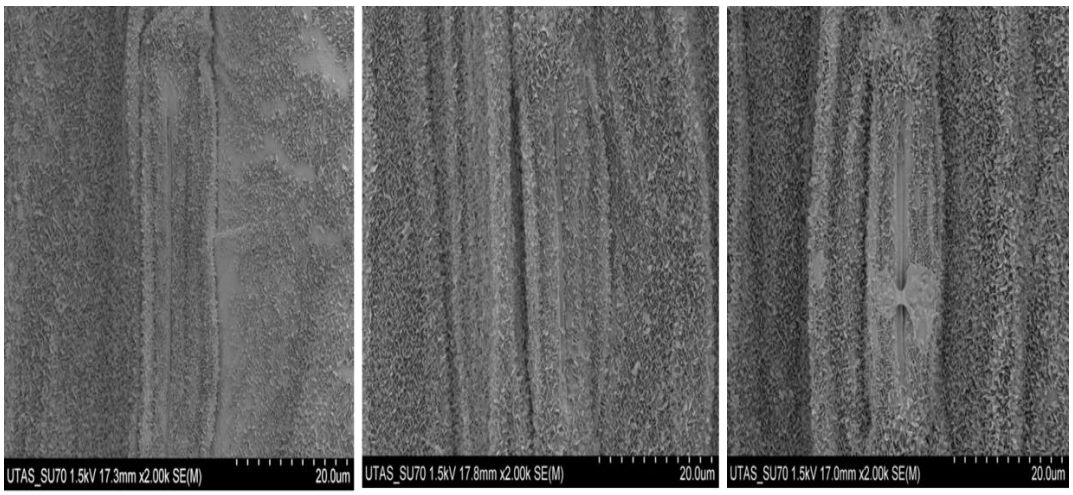


Fig. 4.5 Representative SEM images showing cuticular wax on the adaxial surface in leaves of three different positions in variety Franklin grown under control conditions. One (of six) typical images is shown for each position.

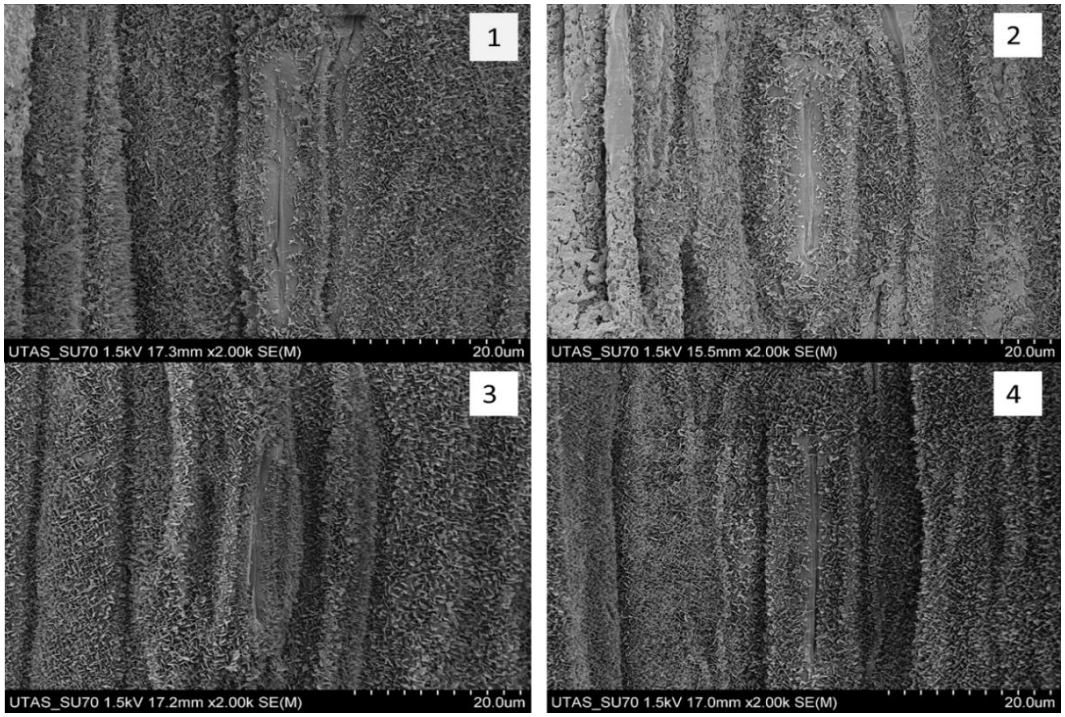
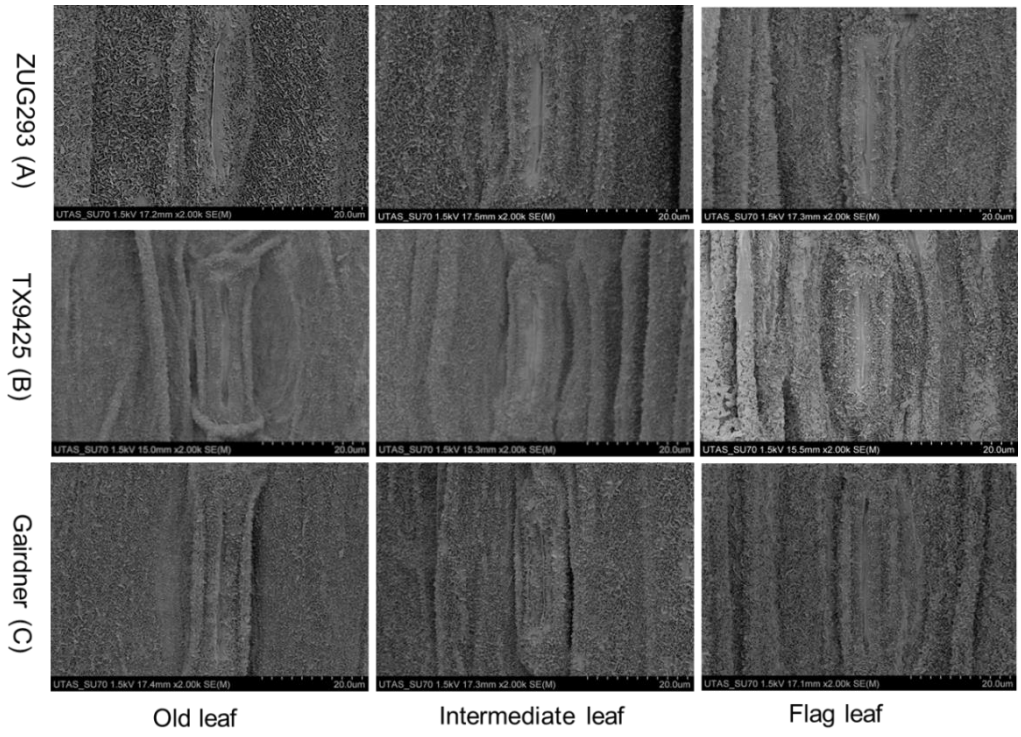


Fig. 4.6 Representative SEM images showing cuticular wax on the adaxial surface of the flag leaf in barley varieties ZUG293 (1), TX9425 (2), Franklin (3) and Gairdner (4) grown under control conditions. One (of six) typical images is shown for each genotype.



Suppl. Fig. 4.1 SEM images showing cuticular wax on the adaxial surface in three different positions of leaf in varieties ZUG293 (A), TX9425 (B) and Gairdner (C) grown under control conditions.

4.3.5 Total wax content of leaves correlates negatively with residual transpiration

A significant negative correlation (R^2 = -0.41 for Method-1 and -0.34 for Method-2; significant at $P < 0.05$) was found between the total cuticular wax content of leaves and residual transpiration measured by two different methods in plants grown under normal growth conditions (Fig. 4.7a).

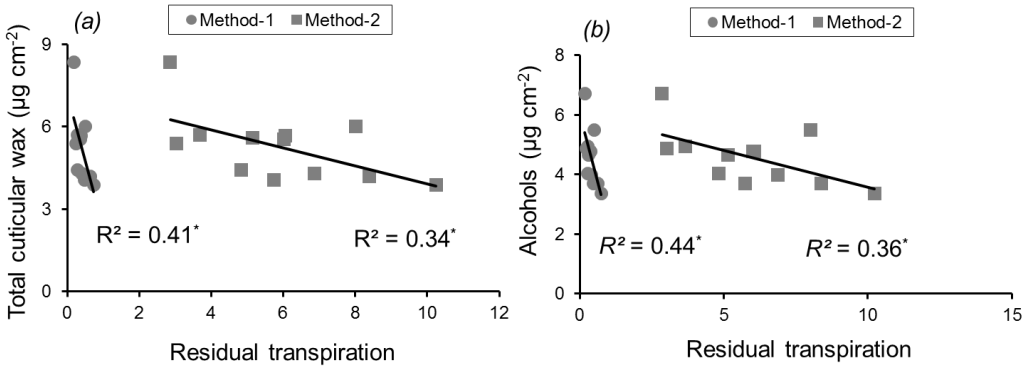


Fig. 4.7 (a) correlations (Pearson's R^2 values) between total cuticular wax and residual transpiration measured by Method-1 (mg H_2O cm^{-2} h^{-1}) and Method-2 (mg H_2O cm^{-2}). (b) correlations (Pearson's R^2 values) between alcohols and residual transpiration measured by Method-1 (mg H_2O cm^{-2} h^{-1}) and Method-2 (mg H_2O cm^{-2}). Data labelled with asterisk are significant at $P < 0.05$.

4.3.6 Cuticular wax constituents, contents and effect on residual transpiration

Across all four barley varieties the average of total leaf cuticular wax was found to be 5.37µg cm^{-2} under normal growth condition. The averages of total cuticular wax of old leaves, intermediate leaves and flag leaves of all genotypes studied were 5.06 µg cm^{-2}, 5.06 µg cm^{-2} and 5.98 µg cm^{-2}, respectively. Cuticular waxes on barley leaves were dominated by primary alcohols (84.7-86.9%), aldehydes (8.90-10.1%), *n*-alkanes (1.31-1.77%), benzoate esters (0.44-0.52%), a phytol related compound (0.22-0.53%), fatty acid methyl esters (0.14-0.33%), β-diketones (0.07-0.23%) and alkylresorcinols constituents (1.65-3.58%). Primary alcohols consisted of odd and even numbers of carbon from C_{22} to C_{29}, particularly *n*-docosanol (C_{22}), *n*-tetracosanol (C_{24}), *n*-hexacosanol (C_{26}), and *n*-octasonanol (C_{28}), and much smaller amount of odd numbered carbons. The higher *n*-alkane component on barley leaf consisted mainly of *n*-hentriacontane

(C_{31}) and *n*-tritriacontane (C_{33}). The main aldehydes were *n*-hexacosanal (C_{26}), *n*-octacosanal (C_{28}) and *n*-triacontanal (C_{30}). Benzoate esters included *n*-docosyl benzoate (C_{22}), *n*-tetracosyl benzoate (C_{24}) and *n*-hexacosyl benzoate (C_{26}). Major fatty acid methyl esters were methyl *n*-octacosanoate (C_{28}), methyl *n*-triacontanoate (C_{30}) and methyl *n*-dotriacontanoate (C_{32}).

Table 4.1 Absolute amount (µg cm^{-2}) of different compounds of cuticular wax on old leaf position of four barley genotypes grown under normal growth conditions (n = 4)

Compound	Genotype				
	ZUG293	TX9425	Franklin	Gairdner	Average
Alcohols	5.48 ± 0.16	3.35 ± 0.65	4.76 ± 0.55	3.99 ± 0.27	4.40
Aldehydes	0.38 ± 0.06	0.46 ± 0.09	0.64 ± 0.06	0.32 ± 0.01	0.45
Alkanes	0.07 ± 0.01	0.08 ± 0.01	0.07 ± 0.01	0.05 ± 0.00	0.07
Benzoate esters	0.02 ± 0.00	0.03 ± 0.01	0.03 ± 0.00	0.01 ± 0.00	0.02
Phytol related	0.01 ± 0.00	0.03 ± 0.01	0.04 ± 0.00	0.02 ± 0.00	0.03
Methyl esters	0.03 ± 0.01	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.01
Diketones	0.01 ± 0.00	0.02 ± 0.01	0.00 ± 0.00	0.00 ± 00	0.01
Alkylresorcinols	0.15 ± 0.05	0.00 ± 0.00	0.15 ± 0.06	0.04 ± 0.01	0.09

Old leaves for all genotypes studied showed the average highest absolute amount of alcohols (4.39 µg cm^{-2}) followed by aldehydes (0.45 µg cm^{-2}) and the lowest β-diketones (Table 4.1). Similar results were found at intermediate and flag leaves for all genotypes (Table 4.2 and 4.3). Among the genotypes, ZUG293 old leaves contained the highest amount of alcohols followed by Franklin. The same results were found for intermediate leaf for all genotypes (Table 4.2). For flag leaves of all genotypes the average highest alcohols were measured from Franklin followed by ZUG293 (Table 4.3).

Table 4.2 Absolute amount (μg cm^{-2}) of different compounds of cuticular wax on intermediate leaf position of four barley genotypes grown under normal growth conditions (n = 4)

Compound	Genotype				
	ZUG293	TX9425	Franklin	Gairdner	Average
Alcohols	4.78 ± 0.08	3.69 ± 0.44	4.65 ± 0.29	4.02 ± 0.32	4.29
Aldehydes	0.41 ± 0.02	0.45 ± 0.06	0.65 ± 0.07	0.38 ± 0.03	0.47
Alkanes	0.06 ± 0.01	0.06 ± 0.00	0.07 ± 0.01	0.05 ± 0.00	0.06
Benzoate esters	0.03 ± 0.00	0.03 ± 0.01	0.04 ± 0.01	0.02 ± 0.00	0.03
Phytol related	0.04 ± 0.01	0.01 ± 0.00	0.03 ± 0.00	0.01 ± 0.00	0.02
Methyl esters	0.00 ± 0.00	0.01 ± 0.00	0.00 ± 0.00	0.02 ± 0.00	0.01
Diketones	0.01 ± 0.00	0.01 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.01
Alkylresorcinols	0.45 ± 0.01	0.03 ± 0.01	0.21 ± 0.06	0.04 ± 0.01	0.18

Table 4.3 Absolute amount (μg cm^{-2}) of different compounds of cuticular wax on flag leaf position of four barley genotypes grown under normal growth condition (n = 4)

Compound	Genotype				
	ZUG293	TX9425	Franklin	Gairdner	Average
Alcohols	4.93 ± 0.21	3.68 ± 0.41	6.71 ± 0.41	4.88 ± 0.17	5.05
Aldehydes	0.40 ± 0.02	0.32 ± 0.05	1.26 ± 0.12	0.45 ± 0.03	0.61
Alkanes	0.06 ± 0.01	0.06 ± 0.00	0.11 ± 0.01	0.06 ± 0.00	0.07
Benzoate esters	0.03 ± 0.00	0.02 ± 0.01	0.05 ± 0.00	0.02 ± 0.00	0.03
Phytol related	0.02 ± 0.00	0.01 ± 0.00	0.02 ± 0.00	0.01 ± 0.00	0.02
Methyl esters	0.01 ± 0.00	0.02 ± 0.00	0.00 ± 0.00	0.05 ± 0.00	0.02
Diketones	0.01 ± 0.00	0.02 ± 0.00	0.01 ± 0.00	0.02 ± 0.00	0.02
Alkylresorcinols	0.36 ± 0.01	0.03 ± 0.00	0.28 ± 0.00	0.04 ± 0.00	0.18

A negative significant correlation (R^2 = -0.44 for Method-1; P < 0.05 and R^2 = -0.36 for Method-2; significant at P < 0.05) was found between residual transpiration and primary alcohols of cuticular wax component of barley genotypes (Fig. 4.7b). No significant correlations were found between residual transpiration measured by two different methods and other cuticular wax components (Table 4.4).

Table 4.4 Correlations (Pearson's R^2 values) between residual transpiration measured by two different methods and different cuticular wax compounds of three different leaf positions of four barley genotypes grown under normal growth conditions. Values labelled with asterisk are significant at P < 0.05

Compound	R^2 values with residual transpiration				Correlation
	Method-1		Method-2		
	R^2 Value	P value	R^2 value	P value	
Aldehydes	0.21	0.15	0.17	0.18	Negative
Alkanes	0.00	0.93	0.00	0.88	Negative
Benzoates	0.16	0.21	0.15	0.21	Negative
Phytols	0.00	0.86	0.05	0.50	Positive
Methyl esters	0.02	0.63	0.06	0.43	Negative
Diketones	0.04	0.52	0.00	0.89	Positive
Alkylresorcinols	0.21	0.15	0.16	0.19	Negative

4.4 Discussion

4.4.1 Residual transpiration and plant water relations

To maintain proper growth and leaf expansion, the growing shoot needs to maintain positive turgor which can be achieved by maintaining osmotic cellular adjustment by either increasing the production of compatible solutes or inorganic ions. As plants accumulate more organic osmolytes in young leaves than old leaves to maintain turgor pressure (Puniran-Hartley et al., 2014), it was

hypothesised that residual transpiration should be less in young leaves due to the fact that they have higher osmolality and hence better water retention, and this was found to be the case. As shown in Fig 4.2a and 4.2b, young flag leaves had a higher osmolality than the older leaves, and increased osmolality had a strong negative correlation with the residual transpiration under normal growth conditions indicating that the increase of leaf sap osmolality might decrease the water transpiration through plant cuticle. An effective osmotic adjustment mechanism may maintain water status in the leaf tissue by decreasing in the cell sap osmotic potential resulting from a net increase of intracellular solutes (Silva et al., 2013).

A leaf can increase its resistance to dehydration through a reduction in cellular osmotic potential by a net accumulation of cellular solutes. In this study, young flag leaves possessed significantly lower osmotic potential than the intermediate and older leaves; a trend that was correlated positively with residual transpiration (Fig. 4.3a and 4.3b). This indicated that a leaf with lower osmotic potential had more turgor pressure to spend and could resist greater loss of water through the cuticle. Lower negative leaf water potential was measured with increasing leaf age for all varieties, which was negatively correlated with residual transpiration (Fig. 4.4a and 4.4b). Young leaves maintained less turgor at more negative leaf water potentials and tended to have less residual transpiration. Increased turgor in the epidermis stretches cuticles and causes a change in gas exchange of the cuticle. A leaf with less turgor would have a tighter cuticle, thus inhibiting gas exchange (Boyer, 2015). Burghardt and Riederer (2003) observed that cuticle gas exchange was affected when leaf water potentials decreased. Thus, leaf water potential affects the diffusion of water vapour through the cuticular barrier, and residual transpiration is negatively correlated with lower leaf water potential (Boyer, 2015) .

4.4.2 Change in residual transpiration to improve water use efficiency

Salinity stress is often referred to as a ‘physiological drought”, so some correlation between salinity and drought stress tolerance is expected. The most salinity tolerant varieties showed the highest residual transpiration under unstressed conditions (Fig. 4.1d). Being somewhat counterintuitive, this is in a

good agreement with Bengston et al. (1978) who showed that drought stress resistant oat genotypes generally transpired the highest amount of water through the cuticle under unstressed conditions, whereas it was strongly reduced under stress conditions. In addition, higher (33 to 38%) residual transpiration in wheat and cotton leaves was reported from irrigated than rainfed field-grown wheat plants (Clarke et al., 1991). On the other hand, deposition of cuticular waxes increased in tolerant genotypes during prolonged drought stress, leading to a reduced rate of residual transpiration (González and Ayerbe, 2010; Shepherd and Wynne Griffiths, 2006).

Water use efficiency can be expressed as the ratio of leaf net carbon assimilation to total transportation water loss. Plants exhibit higher water use efficiency with higher CO_2 assimilation than the stomatal conductance, when non-stomatal water loss is negligible (Yoo et al., 2009). Salt tolerant genotypes transpired more water through cuticle under well irrigated conditions that reveals their water use efficiency is lower than sensitive genotypes. Generally stress tolerant barley genotypes have a lower biomass and yield performance under control conditions (Munns et al, 2006). This could be due to their higher non-stomatal transpiration under irrigated conditions resulting in lower water use efficiency. Conversely, tolerant genotypes could reduce residual water loss under water deficit conditions when stomata are closed and partially closed or both, and this increased water use efficiency could be a significant factor determining their survival capacity to hostile environmental conditions compared to the standard cultivated genotypes. It has been documented that wheat genotypes having lower residual transpiration adapted and performed better under water stress conditions (David, 2010). Genotypes with normally low residual transpiration are at a functional advantage in water-limited environments since they make more efficient use of the water available. Thus, under conditions of water deficit, residual conductance to water vapour may be an important determinant of plant water balance and stress reactivity.

On the other hand, transpiration is the most effective way of leaf cooling of well-irrigated plants. In plants with adequate water supply stomata may regulate leaf temperature close to the optimum for metabolic processes, including photosynthesis or to prevent tissue heat damage under excessive radiation or

temperature (Chaves et al., 2016). Moreover, under water limited conditions, stomatal closure and decreased transpiration, associated with high water use efficiency, may lead to a dramatic increase in leaf temperature (up to 7 ◦C above air temperature) (Blum, 2015). At this condition, high temperatures may disrupt the photosynthetic-related enzymes and produce reactive oxygen species which would challenge the plant cell (Shabala and Munns, 2012).

4.4.3 Relationship between residual transpiration and amounts of cuticular waxes

Our working hypothesis in this study was that reduced residual transpiration should be positively correlated with hydrophobicity of the leaf surface (hence, amount of cuticular waxes deposited). A significant negative correlation (Fig. 4.7a) between the total amount of cuticular wax and residual transpiration was found in the present investigation, which indicated that amount of cuticular wax may create a protecting barrier to reduce the loss of water through the cuticle. Previous studies have reported a weak but significant negative correlation between the cuticular wax and residual transpiration in sorghum (Jordan et al., 1984), wheat (Premachandra et al., 1992), and barley (Larsson and Svenningsson, 1986). This weak correlation may be due to the protecting barrier to the diffusion of water through the cuticle depends on the structure, orientation of wax plates on epidermis, variation of epicuticular and intracuticular wax compositions and distribution of wax plates. Both intracuticular (Zeisler and Schreiber, 2016) and epicuticular (Jetter and Riederer, 2016) wax layer may contribute to the formation of residual transpiration barrier depending on the plant species and cuticle constituents. Plants generally exhibited a significant increase in the amount of cuticular wax amount per unit area of leaves under different stress condition such as water deficit and salinity (Sánchez et al., 2001). The quantity of cuticular wax, however, is not the sole contributor to residual transpiration due to the complexity of water flow through the cuticle (Ristic and Jenks, 2002).

Cuticular waxes have different types of structural morphology including granules, filaments, plates and tubes (Riederer and Muller, 2008). According to the SEM images analysis, plate type cuticular wax observed on the leaf surface consisted of aliphatic compounds in which the primary alcohols *n*-hexacosanol and *n*-

octacosanol were predominant in different leaf positions for all the barley genotypes.

Cuticular waxes on barley leaves consisted of alcohols, aldehydes, alkanes, benzoate esters, phytol related compounds, fatty acid methyl esters, β-diketones and alkylresorcinols (Table 4.1, Table 4.2 and Table 4.3). Generally, plate type primary alcohol based cuticular waxes always dominate on the leaf surface in the Fabaceae and Poaceae (wheat, barley) (Larsson and Svenningsson, 1986) and constitute the major barrier to water loss. This was also the case in our study reported here (Fig. 4.7b) (Ristic and Jenks, 2002). However, such findings could be not generalized to all species. The hydrophobic long chain alcohol, hydrocarbon and aldehyde fractions are the active components of cuticle in controlling residual transpiration in different plant species (Jetter and Riederer, 2016). The main portion of the transpiration barrier in tomato fruits and *Rhazya stricta* leaves is located in the intracuticular wax layer containing large amount of pentacyclic triterpenoids whereas cuticular very long chain aliphatics play a minor role (Schuster et al., 2016; Vogg et al., 2004). Plant species containing fatty acid with very long aliphatic chain (alcohols, aldehydes and alkanes) in the epicuticular wax, together with high amount of alicyclic compounds such as triterpenoids, steroids, or tocopherols in the intracuticular wax contribute equally to the formation of residual transpiration barrier (Jetter and Riederer, 2016). In general, it is accepted that higher levels of long chain aliphatic components in the wax can lead to a higher hydrophobicity of the residual transpiration barrier and thus decrease cuticular water loss (Macková et al., 2013). This should be kept in mind while targeting this trait in the breeding programs.

4.5 Conclusions

Both leaf osmotic potential and the amount of cuticular waxes are involved in controlling water loss from barley leaves under well irrigated conditions. A significant and negative relationship between the amount of primary alcohols and cuticular transpiration implies that primary alcohols may influence the water barrier more than other constituents on plant leaf surface and thus contribute to salinity stress tolerance, at least in barley.

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Chapter 5. Factors determining stomatal and non-stomatal (residual) transpiration and their contribution towards salinity tolerance in contrasting barley genotypes[4]

Abstract

Eighty barley (*Hordeum vulgare* L.) genotypes of different geographical origin and contrasting in salinity stress tolerance were grown under glasshouse conditions and exposed to high salinity stress (300 mM NaCl) for four weeks to investigate the relationship between leaf gas exchange, tissue ionic relations, and plant salinity tolerance. Four weeks after the treatment commenced, stomatal conductance, stomatal density, residual transpiration, chlorophyll content, leaf sap Na, K, Cl and leaf sap osmolality were measured. Responses to salinity stress differed greatly among the genotypes. The overall salinity tolerance significantly correlated with leaf Na^+ content, osmolality and the residual transpiration. At the same time, no significant correlation between salinity tolerance and stomatal conductance was found. The residual transpiration in stressed plants correlated negatively with the leaf sap osmolality of control plants. A significant correlation was found between changes in the residual transpiration and changes in leaf Cl^- content but no such correlation was found for leaf Na^+. Higher stomatal density was correlated with higher osmolality under both salinity stress and control conditions. The stomatal density correlated negatively with the residual transpiration under salinity stress conditions but positively with K^+ accumulation in the shoot under both control and salinity stress conditions. Higher relative stomatal conductance correlated with higher residual transpiration and lower stomatal density under salinity stress. Interestingly, stomatal conductance correlated very strongly with Cl^- accumulation in the shoot under stress conditions but negatively under control conditions. Taking together, these results suggest that increasing stomatal density as well as minimization of the residual transpiration

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may be a promising way of improving water use efficiency and increase salinity tolerance in barley. Our data also show that residual transpiration is strongly affected by the number of stomatal pores on the leaf surface.

5.1 Introduction

Salinity is one of the most common environmental stresses constraining agricultural crop production in the 21st century. The area of land affected by salinity is increasing day by day and cutting average crop yields by 20% to 50% worldwide, costing approximately $27 billion per year and posing a major risk to food security (Qadir et al., 2014). It has been estimated that worldwide 20% of total cultivated and 33% of irrigated agricultural lands are afflicted by high salinity (Shrivastava and Kumar, 2015). Furthermore, salinization is increasing at the rate of 10% annually in arid and semi-arid regions for various reasons, including low rainfall, high surface evaporation, weathering of native rocks, poor irrigation and drainage systems. On the other hand, world population is projected to increase by 9.7 billion in 2050 and 11.2 billion by 2100 (Alexandratos and Bruinsma, 2012). The urgency of feeding the world's growing population needs of increasing the food production by 70% by 2050. This may only be achieved through development of salt tolerant genotypes of major cereal crops by plant breeding and better adaptation of crops to saline-prone environment.

Salinity inhibits plant growth and performance via multiple mechanisms; one of them is an osmotic stress imposed on roots. The osmotic stress is first sensed by plants during an "osmotic phase" that dominates for a few weeks (Munns and Tester, 2008). This phase is then followed by specific ion toxicity in the shoot. Plants deal with osmotic stress by employing a range of biochemical (*de novo* synthesis of compatible solutes for osmotic adjustment; enzymatic and non-enzymatic antioxidant defence system against ROS), morphological (leaf rolling; deposition of cuticular wax; increasing leaf thickness and succulence) and physiological mechanisms. Amongst the latter, efficient control of stomatal and non-stomatal (residual) transpiration to optimize efficiency of CO_2 assimilation is often named as one of the most crucial features (Shabala et al., 2013).

Previous studies reported a positive correlation between salinity stress tolerances in barley and leaf stomatal conductance (Gs), and suggested that this parameter may be used as an important physiological marker for screening for salinity stress tolerance in cereals (Jiang et al., 2006; Rahnama et al., 2010). However, these results were reported for a very mild stress (33 mM NaCl) (Jiang et al., 2006). This concentration is considered to be below the salinity threshold and is hardly a stress for a species that can produce grain yield at concentrations over 300 mM NaCl (Chen et al., 2007). A more wide study using 46 barley genotypes grown in glasshouse conditions under moderate salinity stress (200 mM NaCl) for 5 weeks has failed to find any association between Gs and salinity tolerance (Zhu et al., 2015). So, it appears that the link between these two characteristics not as straightforward as one may think and may be determined/affected by numerous confounding factors.

Past studies have found a positive correlation between Gs and stomatal density, SD (Franks et al., 2009), and studies on halophytes (Shabala et al., 2012; Orsini et al., 2012) have shown that salt-grown plants may dramatically (as much as 30%; Shabala et al., 2012) reduce their SD when grown under saline conditions. It was argued that this alteration in SD may represent a fundamental mechanism by which plant can optimise water use efficiency, WUE (Shabala et al., 2013). This hypothesis was supported by the follow-up work (Franks et al., 2015) that showed that WUE was increased by ~ 20% in Arabidopsis mutants that had a reduced SD as a result of overexpressing EPF2 (epidermal pattering factor) gene. However, to the best of our knowledge no study has ever linked stress-induced changes in the stomatal density with salinity stress tolerance in glycophyte crop species using sufficiently large number of genotypes. Is stomatal density an important component of salinity tolerance mechanism? What are the factors affecting stomatal characteristics under high salinity stress conditions?

The physiological rationale behind this question is the fact that SD is ultimately related to the non-stomatal (residual) transpiration through the leaf. This residual transpiration (RT) refers to water loss through cuticle of the leaf surface during night when stomata are closed completely and/or partially under well irrigated conditions. However, under stressed environmental conditions, a relatively large

portion of evaporated water may bypass the stomata and occur through the cuticle during daytime, when stomata are closed. Depending on the plant species, RT may account for 5-15% of leaf transpiration, and could be even higher (up to 25-30%) under stressed conditions (Caird et al., 2007). RT involves in a significant water loss without allowing CO_2 uptake by impermeable cuticle of leaf surface, resulting in a major reduction of WUE under osmotic stress conditions (Boyer, 2015). Therefore, a reduction of RT could be a potentially useful mechanism for improving plant performance under stress conditions. Reduced RT has been suggested as a selection trait in cereal genotypes when breeding for osmotic stress conditions (Clarke et al., 1991; Petcu, 2005), and our recent work revealed a sufficient variability in RT amongst barley genotypes (Hasanuzzaman et al., 2017). The current work follows up on that study and investigates the role of the RT in salinity tolerance in barley using a large number of barley genotypes contrasting in salinity stress tolerance. To the best our knowledge, no such large-scale screening has been undertaken before in the literature. Our working hypothesis was that stress-induced changes in the residual transpiration correlate with salinity tolerance, and tolerant varieties will possess more efficient means to reduce the extent of RT when grown under saline conditions.

5.2 Material and methods

5.2.1 Plant material and growth condition

Barley (*Hordeum vulgare* L.) seeds were obtained from the Australian Winter Cereal Collection and from the barley genotype collection of Zhejiang and Yangzhou Universities in China. Eighty barley genotypes were grown in a glasshouse using the Mount Pleasant Laboratory facilities in Launceston, Australia. The experiment was conducted in 2016 (January-September) and the mean daily temperatures were 25°C (in the day) and 15°C (at night). Plants were grown in a 40 L poly (vinyl) chloride (PVC) container (four genotypes per container) filled with the fertilised standard potting mix (Hasanuzzaman et al., 2017). Twelve seeds were planted for each genotype. The emerged seedlings (3 leaf stage) were treated with 300 mM NaCl for 4 weeks. Plants were watered with excessive amounts of salt solution several times per day (run for waste). As a result, the concentration of NaCl in the potting mix was stable and matched that of

the irrigation solution (300 mM NaCl). The experiment was conducted as a complete randomized design, with three replications for each cultivar for each of the salinity and control treatments. Control plants were grown under normal irrigated conditions, with EC ranging 1.2-1.5 dSm^{-1}.

5.2.2 SPAD Measurements

Leaf chlorophyll content was measured as a SPAD index with the Minolta SPAD-502 (Konica Minolta Sensing, Tokyo, Japan). Measurements were taken from the middle of the lamina of the intermediate position leaf for each salt-treated and control conditions. Three replicates with five plants for each cultivar were measured, for each salinity stressed and control treatments.

5.2.3 Residual transpiration measurement

Three fully expanded mature leaves at an intermediate position from each genotype from salinity treated and control plants were selected for sampling. The leaves were excised during daytime and sealed with vacuum grease on the cut end immediately. Then collected leaves were immediately transported to the laboratory and placed in the dark room at 20 ± 1°C and 50% relative humidity for stomata closure. Fresh weights (W_0) were measured by an electronic balance immediately after excision of leaves. The leaves were then weighed at 2, 4 and 6 h (W_2, W_4 and W_6 respectively) intervals. The leaves were then placed in dry oven at 60°C for 24 h and measured the dry weights (W_d). RT was measured per dry weight basis by using the following formula

$$\text{Residual transpiration} = \frac{(W_0 - W_2) + (W_2 - W_4) + (W_4 - W_6)}{3 \times W_d(T_2 - T_1)}$$

Where, T_1-T_2 = time interval between two subsequent measurements (2 h). The microscopic observations showed that stomata were fully closed after 1 h in the dark.

The measured RT was then recalculated per projected leaf area basis and expressed in mg H_2O cm^{-2} h^{-1} as described before (Hasanuzzaman et al., 2017).

5.2.4 Stomatal conductance measurement

Stomatal conductance was measured from the fully expanded leaf using a steady state diffusion leaf porometer (model SC-1, Decagon, Australia). Leaves of intermediate position were used for measurements. Measurements were taken under constant light conditions (artificial light of 900-1000 µmol m^{-2} s^{-1}) in a temperature controlled glasshouse conditions. The sample size for each genotype was 15 (five plants/container × 3 replications for each cultivar), for each of salinity stressed and control treatments.

5.2.5 Stomatal density

Stomatal density in barley leaves from intermediate position were quantified by getting leaf imprints for each of salt-treated and control plants. A thin layer of a nail polish was added onto the abaxial surface of the middle portion of leaves. Once dried, the imprints were peeled off by the fine forceps and placed onto a microscope slide and covered with a cover slip. Imprints were examined microscopically at 20 × magnification. The number of stomata was counted from each field of view and stomatal density (number of stomata per surface area) was calculated. The sample size for each genotype was 18 (three fields of view × two imprints × three replications).

5.2.6 Measurement of leaf osmolality

Three fully-expanded leaves at intermediate position were taken from each genotype for each of the salinity stressed and control plants after 4 weeks of 300 mM NaCl treatment. Leaf samples were placed into 1.5 mL Eppendorf tubes and frozen at -20°C overnight. The frozen samples were thawed and then leaf sap squeezed using a pointed glass rod to extract sap. An amount of 10 µL sap was taken from each sample for measuring leaf osmolality using a vapour pressure osmometer (Vapro model 5520, Wescor Inc., Logan, Utah).

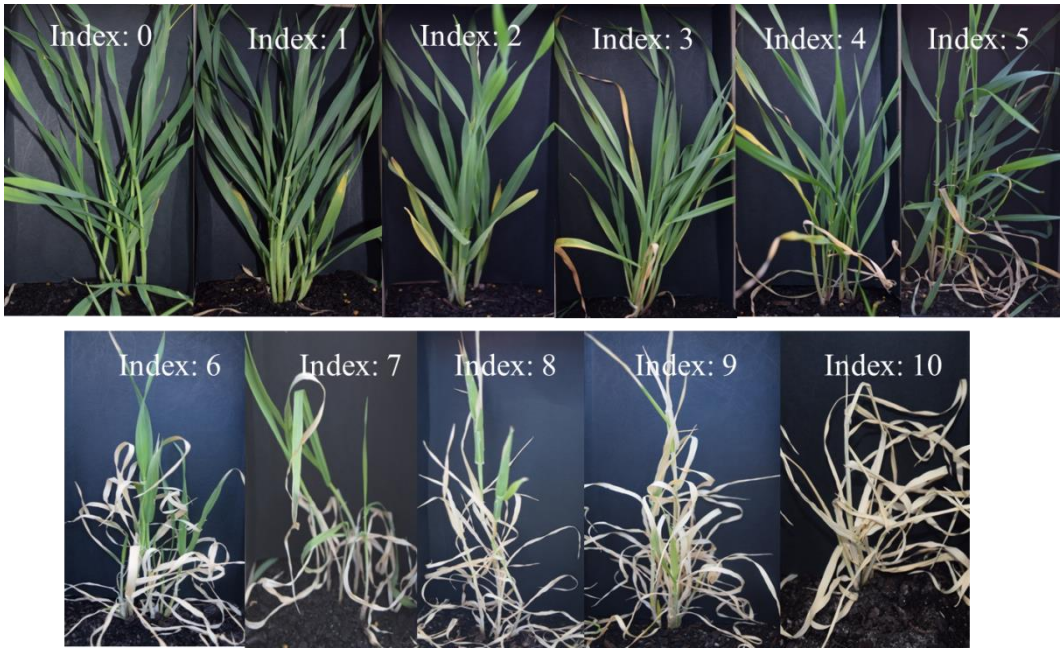
5.2.7 Na^+, K^+ and Cl^- measurements

For the determination of Na^+ and K^+ concentrations, 100 µL of the previously collected leaf sap for osmolality was mixed with 20 mL of distilled water, and the mixture was evaluated by using a flame photometer (PFP7; Jenway, Felsted,

England). Leaf sap chloride content was assessed using Cl^- selective microelectrodes using the Microelectrode Ion Flux Estimation (MIFE) technique (Shabala et al., 2005; Shabala et al., 1997). In brief, microelectrodes with 2-3 µm external tip diameter were pulled from borosilicate glass capillaries (GC150-10; Harvard Apparatus Ltd, Kent, UK) and silanised with tributylchlorosilane (Fluka, Catalogue. No. 90796; Sigma-Aldrich, Steinheim, Switzerland). The electrodes were then back filled with 500 mM KCl adjusted to pH 6 with NaOH and front filled with Cl^- (chloride ionophore I, cocktail A, Fluka 24902, Fluka, Busch, Switzerland). Microelectrodes were mounted on a 3D-micromanipulator (MMT-5; Narishige, Tokyo, Japan) and calibrated in a set of Cl^-standards (250, 500, 1000 µM for control and 250, 1000 and 2000 µM for salt-treated samples). Their tips were then aligned and positioned in a small chamber containing diluted leaf sap. The data were recorded using MIFE CHART software (Shabala et al., 1997) for at least 5 min and Cl^- concentration was determined by taking the mean value of each measurement.

5.2.8 Scoring for salinity stress tolerance

Barley varieties were grown in big (420 L; 1.5 × 0.7 × 0.4 m) PVC tanks filled with the fertilised standard potting mix. Four seeds for each of eighty genotypes were sown in each pot with 6 replications and grown under same glasshouse conditions described above. One week after germination, 300 mM NaCl was gradually added to the irrigation solution over 4 successive days in an attempt to avoid sudden osmotic shock. Salinity treatment continued for 4 weeks, and then the degree of leaf injury and the number of surviving plants were recorded and scored. The extent of leaf injury was then ranked on 0 to 10 scales (0 being no visual symptoms; 10 being dead plants) (Suppl. Fig. 5.1). These scores are termed as ‘salinity damage index’ (SDI) in this work.



Suppl. Fig. 5.1 Visual assessment of salinity tolerance in barley quantified by using leaf injury scoring index. Score 0 was given to plants showing no symptoms of salt injury; score index 10 is given to dead plants. Plants were treated with saline solution (300 mM) till they died.

5.2.9 Statistical analysis

Data were analysed using IBM SPSS Statistics 21 (IBM corp. Armonk, NY, USA) and R version 3.4 (R Core Team, 2017). All results are given as means ± SE. The significance of the correlations between different parameters was determined by bivariate correlations based on Pearson's correlation (two-tailed). Since significant comparisons are expected simply due to chance when multiple tests are conducted, the significances were adjusted for multiplicity using Hochberg's method. A regression model was constructed for salinity damage index using the candidate predictors chlorophyll, stomatal conductance, stomatal density, residual transpiration, osmolality, Na^+, K^+ and Cl^- under saline and control conditions. The model was simplified using stepwise regression in which Akaike information criterion (AIC) was used for selecting the preferred model.

We calculated the ratios of the treatment to the control variables for chlorophyll, residual transpiration, stomatal density, stomatal conductance, osmolality, Na^+, K^+, and Cl^-. We used recursive partitioning (Hothorn et al., 2006) to identify the best of the ratio predictors. This method estimates a regression relationship by binary

recursive partitioning in a conditional inference framework. Essentially it splits up the outcome variable in an optimal way using the explanatory variables. Each split is always significant. This approach attempts to find the "best" model.

5.3 Results

5.3.1 Effect of salinity on leaf chlorophyll and gas exchange characteristics

The effect of high salinity stress (300 mM NaCl) on plant performance was evaluated by the analysis of the changes in SPAD chlorophyll content, residual transpiration, stomatal density, stomatal conductance, osmolality and ion content in tissue (Na, K and Cl) after 4 weeks of salinity treatment relative to controls. SPAD chlorophyll values ranged between 30 ± 0.68 and 44 ± 0.37 in control plants (Fig. 5.1a) and increased in most genotypes under salinity stress conditions (most likely as a result of reduction in the cell size), ranging between 26 ± 0.60 and 55 ± 0.46 (Table 5.1). Only 7 of 80 varieties displayed significant ($P < 0.05$) reduction in the chlorophyll content (Fig. 5.1b). Overall, the relative changes (% of control) differed among genotypes and ranged between 70% for HOR13437 to 137% for HU93-045 (Fig. 5.1b).

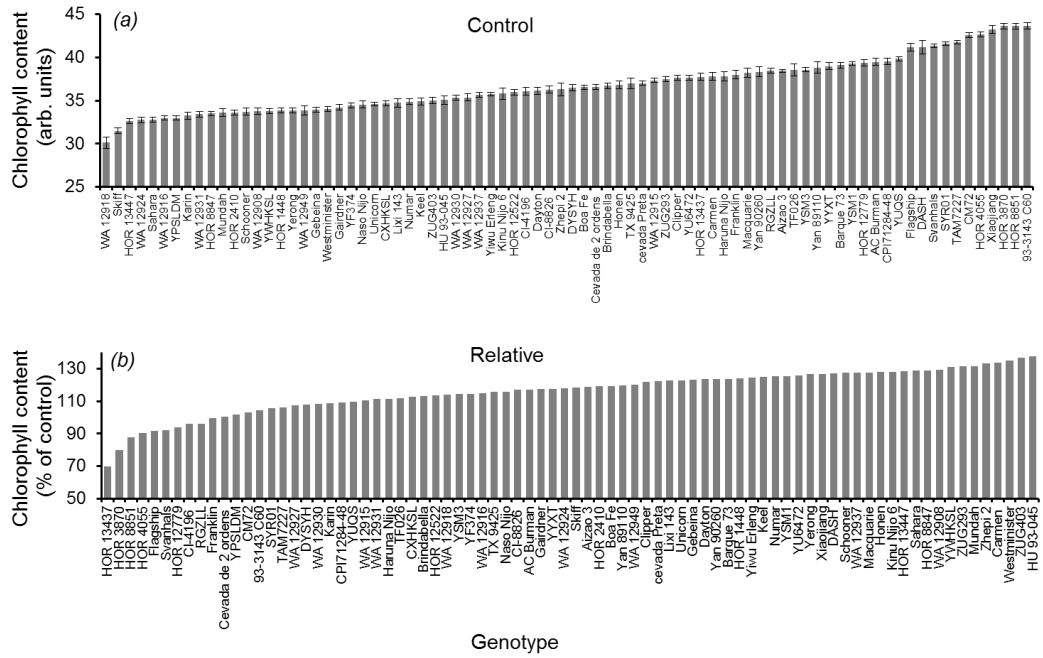


Fig. 5.1 (a) Chlorophyll content (SPAD reading) of 80 barley genotypes grown under control conditions. Data are means ± SE, n = 15. (b) Relative chlorophyll content in salt-affected plants (expressed as a percentage of control).

A significant (nearly 5-fold) variation was found in the RT among the barley genotypes under normal growth conditions, with RT values ranging between 0.27 ± 0.03 mg H_2O cm^{-2} h^{-1} and 1.27 ± 0.09 mg H_2O cm^{-2} h^{-1} (Fig. 5.2a). Four weeks of salinity stress (300 mM NaCl) caused a very significant decrease in the RT, reducing it into 0.13 ± 0.01 to 0.50 ± 0.02 mg H_2O cm^{-2} h^{-1} range (Table 5.1). The relative values of RT of salinity stressed plants (% of control) showed a great extent of genetic variability ranging from 11.9% for genotype Yerong (highest reduction) to 76.4% for genotype Skiff (Fig. 5.2b).

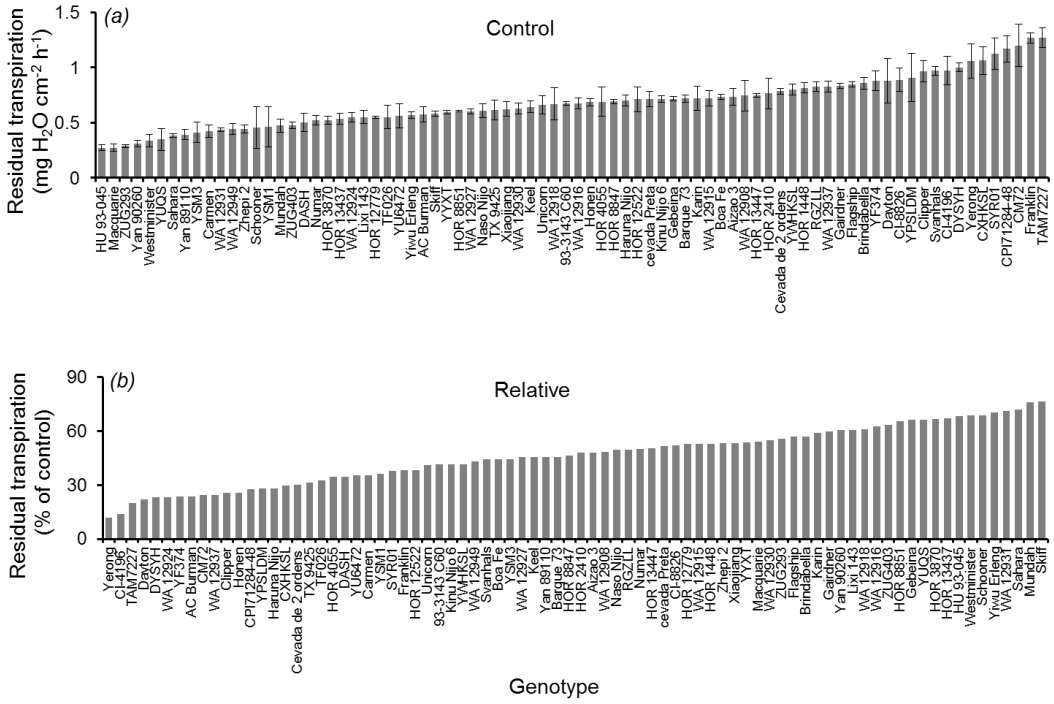


Fig. 5.2 (a) Residual transpiration (mg H_2O cm^{-2} h^{-1}) of 80 barley genotypes under control conditions. Data are means ± SE, *n* = 15. (b) Relative residual transpiration in salt-affected plants (expressed as a percentage of control).

About 2-fold variability was found in the SD among the genotypes under normal irrigated condition (Fig. 5.3a). The lowest SD was found in the genotype HOR 2410 (114 ± 2 cells mm^{-2}). The highest SD was observed in genotypes Macquarie (296 ± 10 cells mm^{-2}). This genotype, however, was somewhat an exception and most other varieties on this end had SD values of around 200 cells mm^{-2} (Fig. 5.3a). The effect of salinity stress on SD was complex (Table 5.1), with about 1/3 of all genotypes showing a significant decline in SD (to as low as 62% of control

in Shahara; Fig. 5.3b), while other 1/3 showed an increase in SD (to as high as 171% in SYR01; Fig. 5.3b).

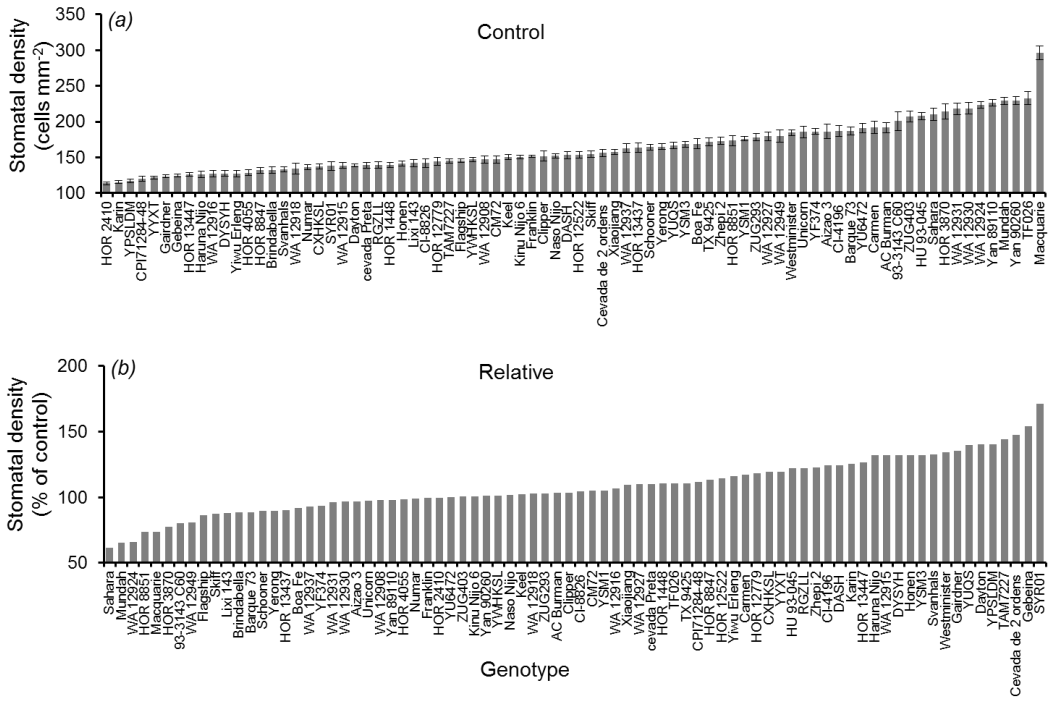


Fig. 5.3 (a) Stomatal density (cells mm^{-2}) of 80 barley genotypes grown under control conditions. Data are means ± SE, n = 18. (b) Relative stomatal density in salt-affected plants (expressed as a percentage of control).

The mean Gs in control was around 150 mmol $m^{-2}s^{-1}$ (Fig. 5.4a) and reduced dramatically in all genotypes grown under salinity stress conditions (300 mM NaCl for 4 weeks) (Table 5.1). The reported range of Gs values in control plants was between 101 ± 5 to 314 ± 10 mmol m^{-2} s^{-1} (Fig. 5.4a). Under salinity stress conditions, Gs ranged between 25 ± 2 mmol m^{-2} s^{-1} (Cl-4196) and 175 ± 9 mmol m^{-2} s^{-1} (Boa Fe) (Table 5.1). The relative changes (% of control) of Gs in salinity stressed plants ranged between 76% for Lixi 143 and as low as 10% for Cl-4196 (Fig. 5.4b).

5.3.2 Plant ionic relations under saline conditions

Leaf sap osmolality ranged between 336 ± 26 to 779 ± 75 mmol kg^{-1} in control (Fig. 5.5a) and increased in all genotypes under high salinity (300 mM NaCl) stress conditions (Table 5.1). Under salinity stress conditions, osmolality ranged between 835 ± 30 (TF026) to 3374 ± 68 (Keel) mmol kg^{-1} (Table 5.1). The

relative changes (% of control) in leaf sap osmolality ranged between 152% (ZUG293) to 684% (WA12927) (Fig. 5.5b).

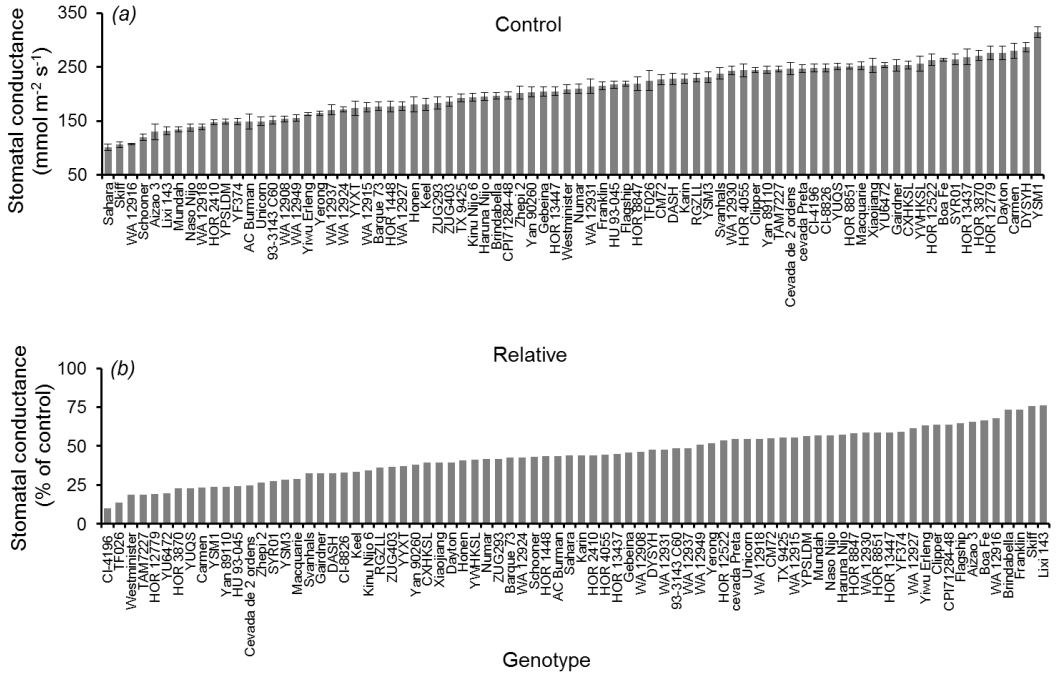


Fig. 5.4 (a) Stomatal conductance (mmol m^{-2} s^{-1}) of 80 barley genotypes grown under control conditions. Data are means ± SE, *n* = 15. (b) Relative stomatal conductance in salt-affected plants (expressed as a percentage of control).

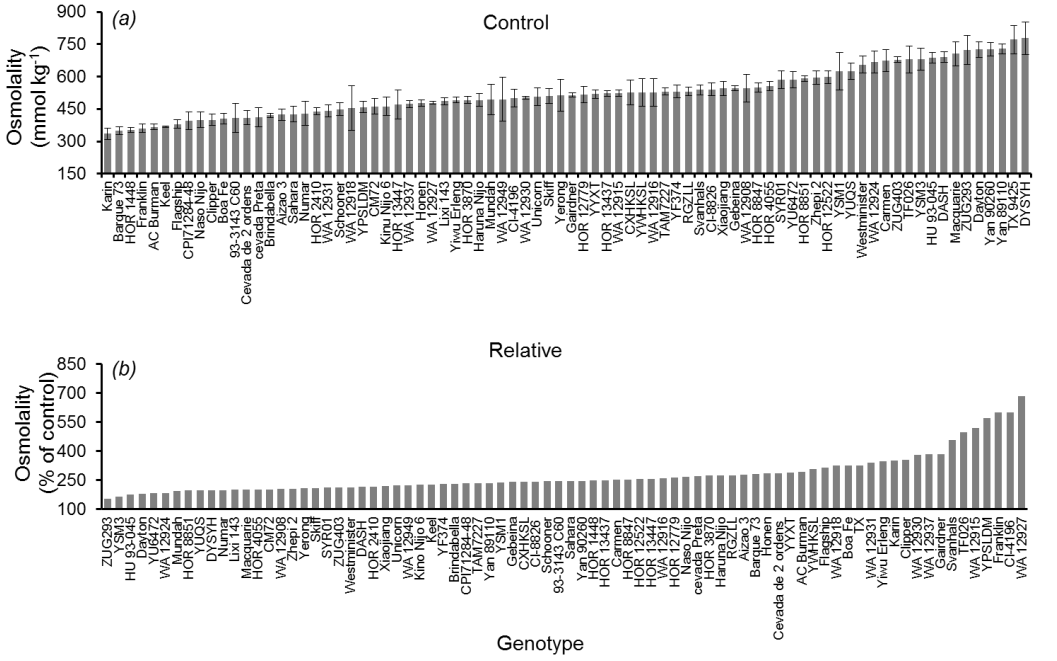


Fig. 5.5 (a) Osmolality (mmol kg^{-1}) of 80 barley genotypes under control conditions. Data are means ± SE, *n* = 15. (b) Relative osmolality in salt-affected plants (expressed as a percentage of control).

Barley plants showed a high variability (8-fold) in the leaf sap Na^+ content under control growth conditions, with Na^+ content in the leaf sap ranging between 10 ± 1 mM in genotype Mundah and 77 ± 12 mM in genotype Cl-4196 (Fig. 5.6a). Salinity stress resulted in a massive accumulation of Na^+ in the shoot, with leaf sap Na^+ content ranging between 149 ± 7 mM in Zhepi2 and 1259 ± 65 mM in genotype Cl-4196 (Table 5.1). The lowest increase in leaf Na^+ was reported in genotype YYXT (595% of control) and the highest in genotype Mundah (4355% of control) (Fig. 5.6b).

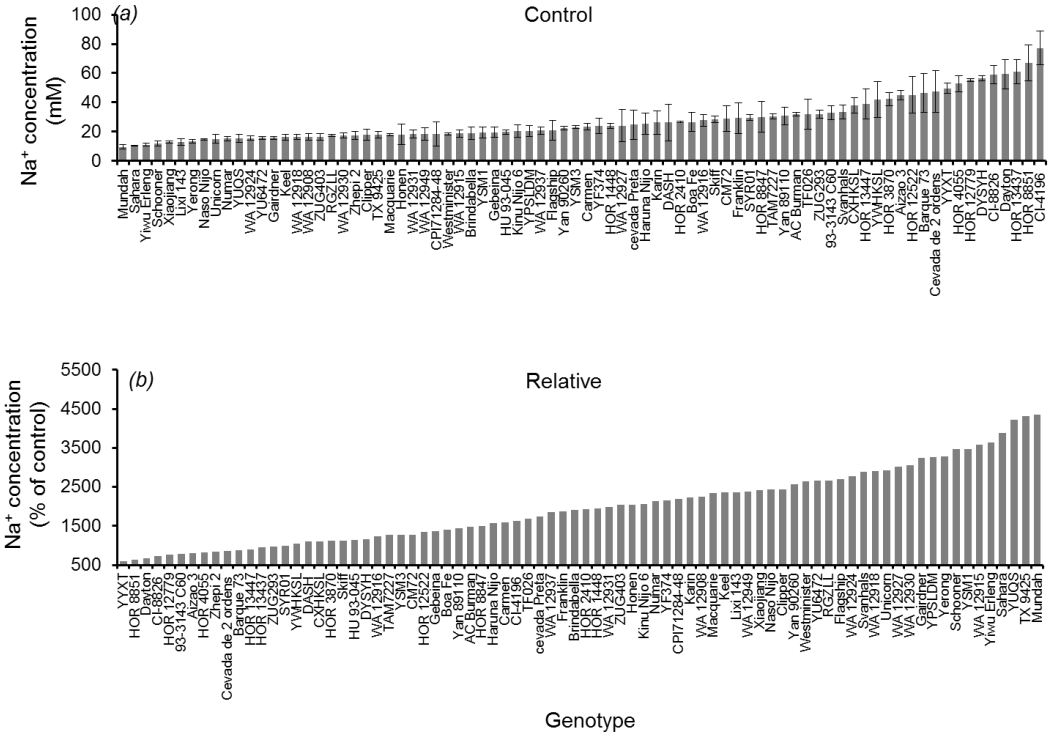


Fig. 5.6 (a) Leaf sap Na^+ concentration (mM) of 80 barley genotypes under under control conditions. Data are means ± SE, n = 15. (b) Relative leaf sap Na^+ concentration in salt-affected plants (expressed as a percentage of control).

K^+ content in the leaf sap ranged between 98 ± 9 mM in Mundah and 238 ± 14 mM in genotype DYSYH under optimum growth conditions (Fig. 5.7a). Four weeks of severe salinity stress reduced K^+ content in the leaf sap of most genotypes to as low as 25 ± 1 mM in genotype WA12918 (Table 5.1). The relative changes (% of control) in K^+ content in the leaf sap ranged between 23% in genotype WA12918 and 174% in genotype TF026 (Fig. 5.7b); however, the

stress-induced significant increase in leaf K^+ content was observed only in 5 out of 80 varieties (Fig. 5.7b).

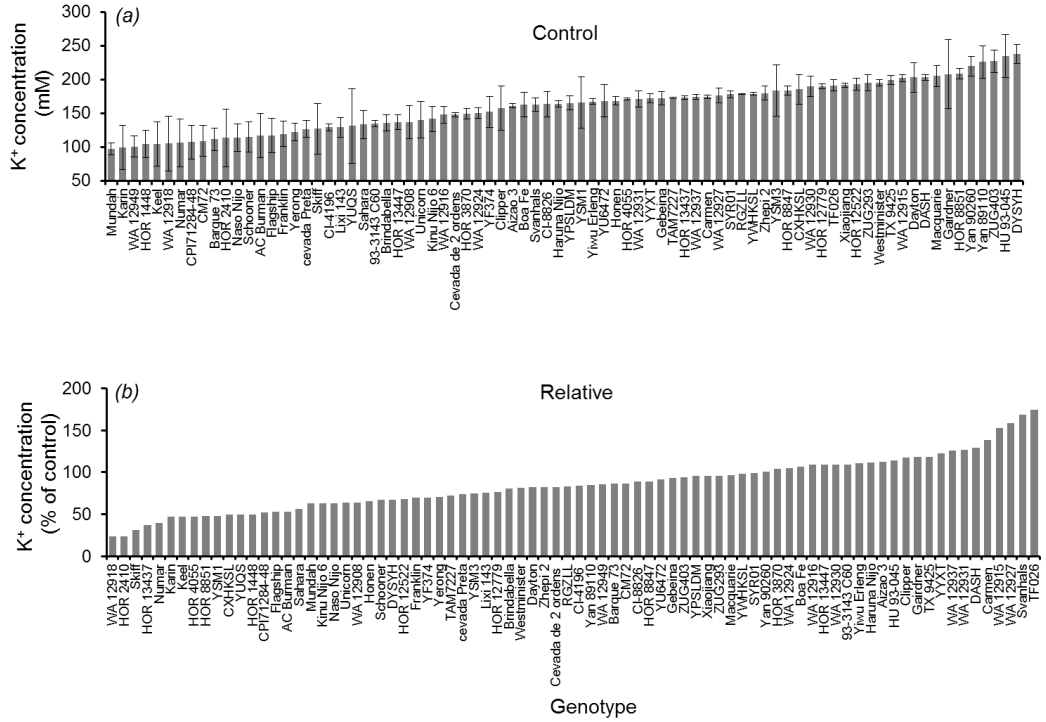


Fig. 5.7 (a) Leaf sap K^+ content (mM) of 80 barley genotypes grown under control conditions. Data are means ± SE, *n* = 15. (b) Relative leaf sap K^+ content in salt-affected plants (expressed as a percentage of control).

Cl^- content in the leaf sap varied between 34 ± 5 mM in genotype YPSLDM and 540 ± 31 mM in genotype HOR8851 under normal growth conditions (Fig. 5.8a) and increased significantly in all genotypes grown under 300 mM NaCl stress. In salt-grown plants, Cl^- content in the leaf sap ranged between 505 ± 61 mM (WA12927) and 1670 ± 108 mM (TAM407227) (Table 5.1). The relative changes (% of control) of Cl^- content of leaf sap showed a 27-fold variability and ranged from only 136% in genotype HOR4055 to 3733% in genotype WA12927 (Fig. 5.8b).

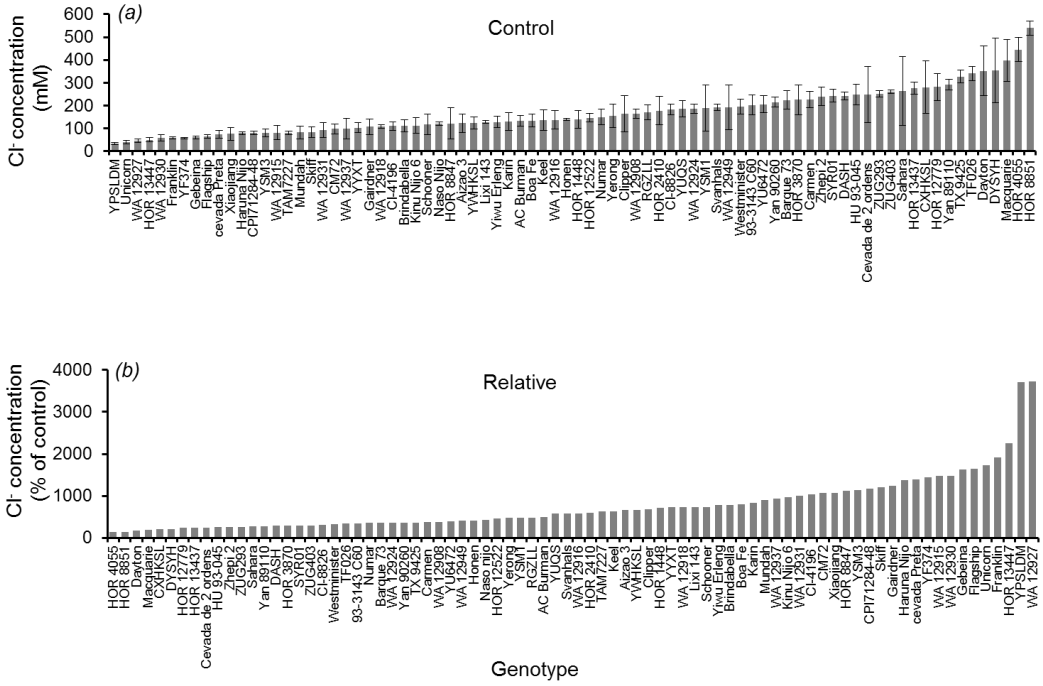


Fig. 5.8 (a) Leaf sap Cl^- content (mM) of 80 barley genotypes grown under control conditions. Data are means ± SE, *n* = 15. (b) Relative Leaf sap Cl^- content in salt-affected plants (expressed as a percentage of control).

Table 5.1 Chlorophyll content, residual transpiration, stomatal density, stomatal conductance, osmolality and leaf sap Na^+, K^+ and Cl^- content in leaf tissue of 80 barley genotypes grown under 300 mM NaCl for 4 weeks.

Genotype	Chlorophyll content (arb. Units)	Residual transpiration (mg H_2O cm^{-2} h^{-1})	Stomatal density (cells mm^{-2})	Stomatal conductance (mmol m^{-2} s^{-1})	Osmolality (mmol kg^{-1})	Leaf sap Na^+ content (mM)	Leaf sap K^+ content (mM)	Leaf sap Cl^- content (mM)
93-3143 C60	46±0.48	0.28±0.02	152±3	73±3	992±24	259±56	148±18	702±40
AC Burman	46±0.43	0.14±0.02	194±4	65±3	1078±28	475±10	62±6	659±27
Aizao3	46±0.28	0.35±0.06	173±4	85±4	1183±82	361±80	182±13	813±70
Barque73	48±0.31	0.33±0.04	164±4	76±3	987±35	407±35	97±10	805±12
Boa Fe	44±0.35	0.32±0.01	155±6	175±9	1319±95	373±68	174±23	1064±91
Brindabella	42±0.41	0.49±0.03	114±6	144±4	968±12	360±24	110±4	871±19
cevada Preta	45±0.31	0.37±0.02	151±6	134±6	1109±95	430±35	94±7	1037±72
Clipper	46±0.30	0.25±0.03	148±6	156±6	1417±93	436±62	185±23	1113±102
CM72	44±0.27	0.29±0.04	154±7	125±2	929±39	368±27	94±37	1037±85
CPI71284-48	43±0.19	0.32±0.07	132±2	126±3	924±11	404±20	56±2	921±44
Flagship	38±0.14	0.48±0.05	125±2	141±7	1202±96	562±96	62±3	1050±78
Franklin	38±0.12	0.48±0.07	150±5	158±7	2161±98	550±96	83±18	1104±108
Gairdner	40±0.40	0.50±0.02	166±6	82±4	1975±85	515±116	246±32	1322±103
Gebeina	42±0.56	0.47±0.08	191±4	93±2	1310±77	269±26	160±4	970±48
Haruna Nijo	42±0.44	0.39±0.07	163±3	112±4	1340±43	401±29	183±8	1094±52
HOR13447	42±0.31	0.38±0.08	159±7	121±2	1209±81	350±75	149±18	1108±109
HOR1448	42±0.22	0.43±0.08	153±4	77±4	877±35	468±28	52±7	998±22
HOR2410	40±0.41	0.37±0.09	114±3	65±3	944±13	510±29	27±1	1055±59
HOR8847	43±0.37	0.32±0.04	148±2	128±8	1383±11	455±3	164±8	1351±9
Karin	36±0.55	0.42±0.05	143±5	100±3	1176±90	584±55	47±12	1092±127
Keel	44±0.41	0.29±0.04	152±3	61±2	835±30	385±12	50±6	860±42
Kinu Nijo6	46±0.44	0.30±0.02	151±2	67±3	1048±27	420±50	90±12	1080±58
Lixi143	43±0.27	0.33±0.05	123±4	101±3	964±11	307±31	98±2	934±31
Naso Nijo	40±0.36	0.30±0.02	154±4	79±2	1066±40	359±58	72±13	510±65
Numar	44±0.29	0.26±0.01	133±4	87±2	848±44	325±45	42±5	530±51
Mundah	44±0.41	0.36±0.04	147±4	76±1	959±42	414±20	62±13	743±56
Sahara	42±0.51	0.28±0.02	129±6	44±2	1038±40	403±86	75±9	708±8
Schooner	43±0.25	0.31±0.03	145±5	51±2	1089±65	412±22	78±11	860±56
Skiff	37±0.41	0.45±0.02	133±6	81±4	1061±50	319±38	40±7	993±71
Unicorn	43±0.27	0.27±0.04	175±5	82±3	1125±63	439±23	90±4	675±36
WA12908	44±0.27	0.36±0.02	141±3	71±3	1106±31	369±106	88±3	621±24
WA12916	38±0.36	0.42±0.08	132±3	73±4	1362±58	343±87	161±9	799±32
WA12918	34±0.29	0.41±0.03	135±4	76±3	1475±48	476±113	25±1	781±32
WA12924	39±0.42	0.13±0.02	146±8	73±2	1217±39	434±15	159±13	668±49
WA12949	41±0.52	0.19±0.03	139±7	79±3	1106±22	438±2	87±8	776±18
Yerong	43±0.22	0.13±0.01	147±4	85±2	1066±65	437±19	86±9	748±24
YF374	39±0.59	0.21±0.04	172±4	88±4	1213±17	516±25	106±3	830±41
YSM1	49±0.40	0.17±0.01	184±3	75±4	1492±96	669±144	80±13	917±174
YSM3	44±0.32	0.18±0.05	220±9	66±2	1108±37	294±17	137±14	913±269
YU6472	47±0.27	0.20±0.03	188±5	49±2	1066±45	422±143	155±33	820±163
YUQS	44±0.22	0.23±0.02	230±7	58±4	1226±52	646±228	65±43	1062±304
Zhepi2	48±0.37	0.24±0.02	208±6	54±2	1213±94	149±7	148±17	640±30
ZUG293	49±0.21	0.16±0.00	181±5	77±3	1095±19	315±12	188±35	674±8
ZUG403	48±0.23	0.30±0.02	206±10	68±3	1442±85	338±33	214±20	778±43
HU93-045	48±0.25	0.19±0.02	253±11	52±2	1212±94	227±22	269±30	656±23
Macquarie	49±0.39	0.15±0.02	216±6	73±4	1412±52	423±31	198±12	772±69
Yan89110	47±0.46	0.18±0.02	218±7	58±4	1712±59	442±80	193±5	783±51
Yan90260	47±0.44	0.19±0.02	233±12	77±4	1776±35	581±24	221±20	784±44
Westminister	46±0.20	0.23±0.02	247±8	39±1	1396±67	488±43	159±2	637±5
DASH	52±0.40	0.18±0.03	189±5	74±3	1472±29	290±68	262±6	695±34
TF026	43±0.56	0.18±0.03	259±27	30±1	3374±68	543±26	334±44	1174±81
TX9425	43±0.59	0.19±0.01	188±9	107±4	2515±97	780±91	237±13	1205±158
Carmen	51±0.62	0.15±0.04	214±9	65±2	1698±75	373±25	242±26	838±26
Cevada de2 ordens	37±0.70	0.24±0.03	223±14	61±2	1173±29	412±18	122±5	600±20
CI-4196	35±0.70	0.14±0.02	227±13	25±2	2991±46	1259±65	108±15	1142±17
CI-8826	42±0.56	0.46±0.02	145±7	81±5	1308±90	425±62	146±14	571±57
CXHKSL	39±0.50	0.32±0.05	162±8	99±6	1266±66	421±66	92±3	583±23
Dayton	45±0.33	0.19±0.05	192±7	109±5	1291±26	399±28	167±14	588±29
DYSYH	39±0.38	0.23±0.06	162±9	136±8	1528±52	659±105	161±20	745±78
Honen	47±0.29	0.18±0.02	184±4	74±6	1365±98	372±57	111±5	565±29
HOR12522	41±0.48	0.27±0.06	174±8	141±7	1535±73	607±73	131±15	677±60
HOR12779	37±0.35	0.29±0.05	167±5	52±2	1358±49	421±103	145±9	677±25
HOR13437	26±0.60	0.36±0.04	146±6	120±7	1304±41	585±25	64±7	666±26
HOR3870	35±0.38	0.35±0.03	163±12	62±3	1338±55	472±24	156±25	651±71
HOR4055	38±0.24	0.24±0.03	124±2	109±7	1115±58	432±24	81±4	606±31
HOR8851	38±0.37	0.40±0.03	125±7	147±4	1156±34	431±6	100±5	797±38
RGZLL	37±0.34	0.41±0.02	166±6	83±2	1461±62	463±73	149±18	829±71
Svanhals	38±0.32	0.43±0.07	176±9	77±2	2469±98	966±150	276±29	1114±219
SYR01	44±0.65	0.43±0.03	231±4	72±5	1226±36	294±60	177±6	728±20
TAM407227	44±0.44	0.25±0.01	209±6	46±2	1242±19	385±42	125±5	505±61
WA12915	41±0.40	0.38±0.03	180±4	97±4	2723±82	669±79	308±16	1188±77
WA12927	38±0.18	0.27±0.03	193±4	109±5	3273±80	729±163	280±17	1670±108
WA12930	38±0.27	0.34±0.02	207±5	143±11	1913±69	532±59	208±5	841±61

WA12931	37±0.36	0.31±0.01	205±6	103±1	1499±67	362±32	218±5	914±30
WA12937	46±0.37	0.20±0.02	149±5	83±2	1806±96	383±114	219±20	919±40
Xiaojiang	55±0.46	0.33±0.02	170±7	99±3	1187±46	313±21	184±13	808±103
Yiwu Erleng	45±0.33	0.40±0.02	145±9	103±5	1709±56	403±99	185±10	1002±34
YPSLDM	34±0.34	0.26±0.05	162±4	84±2	2628±68	672±161	159±12	1256±44
YWHKSL	44±0.41	0.33±0.05	148±5	106±7	1608±83	435±145	176±21	825±126
YYXT	46±0.34	0.32±0.04	145±4	64±3	1505±28	296±58	212±12	744±46

5.3.3 Correlation analysis

Correlations were tested between the extent of leaf injury of salt-grown plants (a measure of plants' salt tolerance; Suppl. Fig. 5.1) and measured physiological (SPAD chlorophyll values, stomatal conductance, stomatal density, residual transpiration, osmolality) and ionic (Na, K and Cl content) characteristics. A significant negative correlation (R^2 = - 0.13; $P < 0.001$) was observed between SPAD chlorophyll values and the overall salinity damage index (Fig. 5.9b), with more tolerant varieties (lower damage index) having higher SPAD values under saline conditions. The salinity damage index was positively correlated (R^2 = 0.10; $P < 0.01$) with Na^+ content in leaf sap (Fig. 5.9c) potentially explaining higher extent of leaf chlorosis in sensitive varieties. A significant positive correlation (R^2 = 0.12; $P < 0.01$) was also found between the osmolality of leaf sap and salinity damage index (Fig. 5.9d), most likely as a result of higher accumulation of Na^+ in sensitive genotypes. However, no significant ($P > 0.05$) correlation was found between Gs in salinity stressed plants and salinity damage index (Fig. 5.9e) suggesting that tissues tolerance mechanisms dominated plant responses to high salinity stress conditions.

The salinity damage index was positively correlated with both absolute RT under salinity stress conditions (R^2 = 0.16; $P < 0.001$) and relative changes (% of control) in RT values (R^2 = 0.10; $P < 0.01$) (Fig. 5.10a and 5.10b). The overall decline in RT values under stress conditions and a strong positive correlation between salt tolerance (lower salinity damage index) and a reduction in RT indicate that all varieties were able to reduce RT under stress conditions, but tolerant genotypes were more efficient in doing this. The RT in stressed plants also showed a significant negative (R^2 = - 0.23; $P < 0.001$) correlation with osmolality in control plants (Fig. 5.10c) and a positive correlation (R^2 = 0.19; $P < 0.001$) with relative changes in leaf sap Cl^- content (Fig. 5.10d). However, no significant correlation ($P > 0.05$) was found between RT under salinity stress conditions and relative

changes (% of control) in leaf sap Na^+ content (Fig. 5.10e). A significant negative correlation (R^2 = - 0.12; P < 0.01) was found between the salinity damage index and the relative changes (% of control) of stomatal density under salinity stress conditions (Fig. 5.10f).

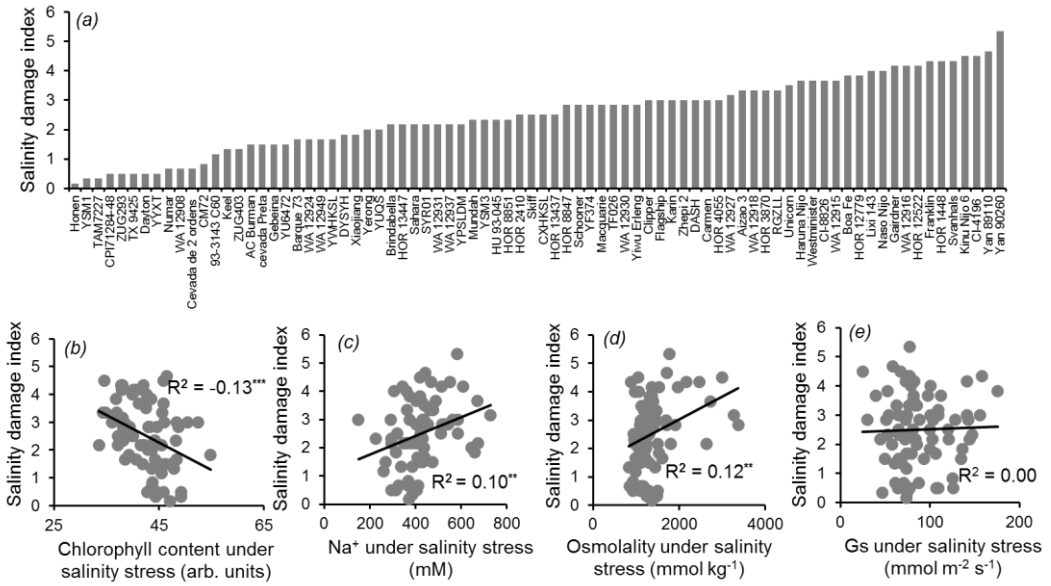


Fig. 5.9 (a) Eighty barley genotypes ranking according to salinity damage index. Plants were exposed to 300 mM NaCl salinity treatment for 4 weeks. Score 0 indicates no visual symptoms of damage; score 10 indicates dead plants. (b) Correlation (Pearson's R^2 value) between chlorophyll content under salinity stress conditions and salinity damage index. (c) Correlation (Pearson's R^2 value) between leaf sap Na^+ content under salinity stress conditions and salinity damage index. (d) Correlation (Pearson's R^2 value) between leaf osmolality under salinity stress conditions and salinity damage index. (e) Correlation (Pearson's R^2 value) between stomatal conductance under salinity stress conditions and salinity damage index. Significant differences for a two-tailed test are indicated: *, P < 0.05; **, P < 0.01; ***, P < 0.001.

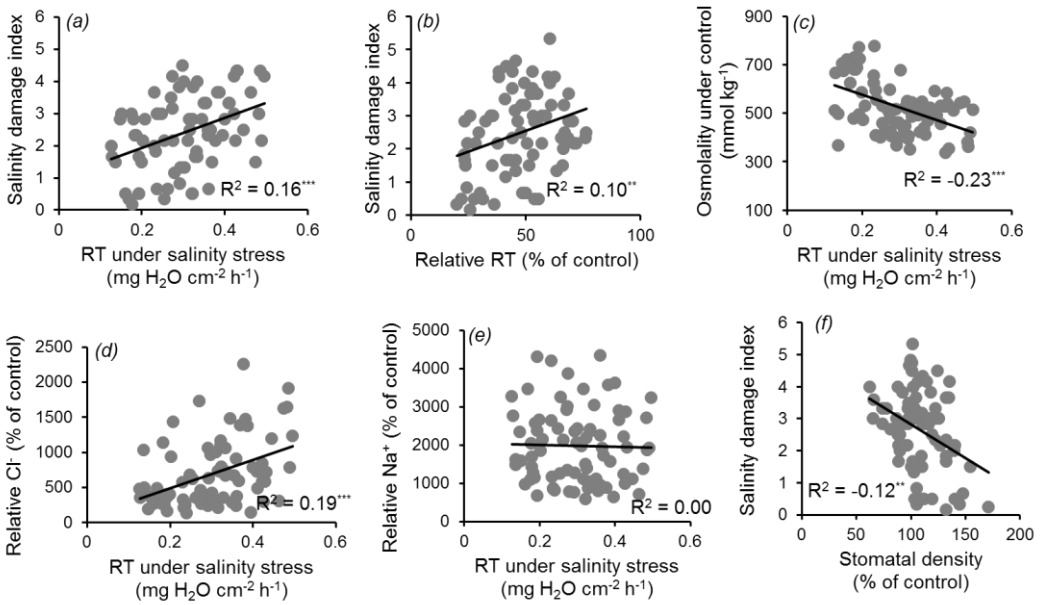


Fig. 5.10 (a) Correlation (Pearson's R^2 value) between the residual transpiration under salinity stress conditions and salinity damage index. (b) Correlation (Pearson's R^2 value) between relative residual transpiration (% of control) under salinity stress conditions and salinity damage index. (c) Correlation (Pearson's R^2 value) between the residual transpiration under salinity stress conditions and leaf sap osmolality under control conditions. (d) Correlation (Pearson's R^2 value) between the residual transpiration under salinity stress conditions and a relative leaf sap Cl^- content (% of control). (e) Correlation (Pearson's R^2 value) between the residual transpiration under salinity stress conditions and a relative leaf sap Na^+ content (% of control). (f) Correlation (Pearson's R^2 value) between the relative changes (% of control) of stomatal density under salinity stress conditions and the salinity damage index. Significant differences for a two-tailed test are indicated: *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

Stomatal density of salt-grown plants correlated positively ($R^2 = 0.29$; $P < 0.001$) with leaf sap osmolality under both control (Fig. 5.11a) and salinity stress ($R^2 = 0.15$; $P < 0.001$) conditions (Fig. 5.11b). At the same time, SD correlated negatively ($R^2 = -0.23$; $P < 0.001$) with RT under salinity stress conditions (Fig. 5.11c). Stomatal density under salinity stress conditions has also showed a significant positive ($R^2 = 0.28$ and 0.16; $P < 0.001$) correlation with leaf sap K^+ content under both salinity stress and control conditions, respectively (Fig. 5.11d and 5.11e), as well as relative changes (% of control) in leaf sap of K^+ content ($R^2 = 0.28$; $P < 0.001$; Fig. 5.11f).

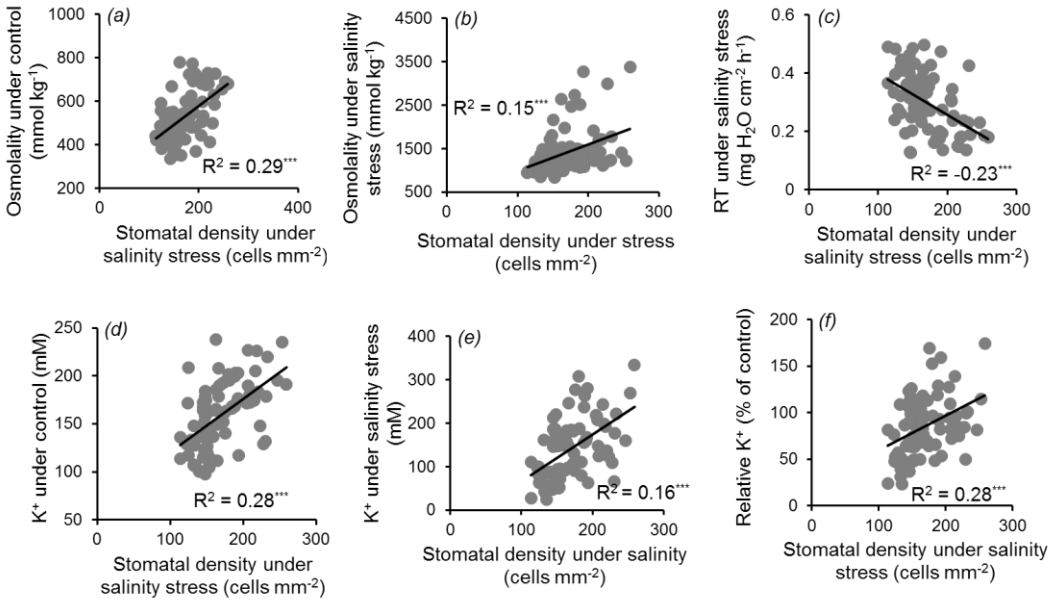


Fig. 5.11 (a) Correlation (Pearson's R^2 value) between the stomatal density under salinity stress conditions and a leaf sap osmolality under control conditions. (b) Correlation (Pearson's R^2 value) between the stomatal density under salinity stress conditions and a leaf sap osmolality under salinity stress conditions. (c) Correlation (Pearson's R^2 value) between the stomatal density under salinity stress conditions and the residual transpiration under salinity stress conditions. (d) Correlation (Pearson's R^2 value) between the stomatal density under salinity stress conditions and a leaf sap K^+ content under control conditions. (e) Correlation (Pearson's R^2 value) between the stomatal density under salinity stress conditions and a relative leaf sap K^+ content (% of control). (f) Correlation (Pearson's R^2 value) between the stomatal density under salinity stress conditions and a leaf sap K^+ content under salinity stress. Significant differences for a two-tailed test are indicated: *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

Relative changes in the Gs were correlated positively ($R^2 = 0.19$; $P < 0.001$) with RT under salinity stress (Fig. 5.12a) suggesting that water may evaporate even via closed stomata. A strong negative correlation ($R^2 = -0.42$; $P < 0.001$) was also found between relative Gs changes and SD under salinity stress (Fig. 5.12b), with plants having higher SD showing bigger reduction in Gs. Relative changes (% of control) in Gs also negatively ($R^2 = -0.12$; $P < 0.01$) correlated with relative changes (% of control) in SD (Fig. 5.12c). The relative changes (% of control) of Gs negatively ($R^2 = -0.25$; $P < 0.001$) correlated with the leaf Cl^- concentration under control conditions (Fig. 5.12d). However, a significant positive ($R^2 = 0.15$; $P < 0.001$) correlation was observed between the relative changes (% of control)

of Gs under salinity stress and leaf Cl^- concentration under salinity stress (Fig. 5.12e). Interestingly, relative changes (% of control) in Gs showed strong positive association (R^2 = 0.22; P < 0.001) with the relative changes in the leaf sap Cl^- concentration (Fig. 5.12f).

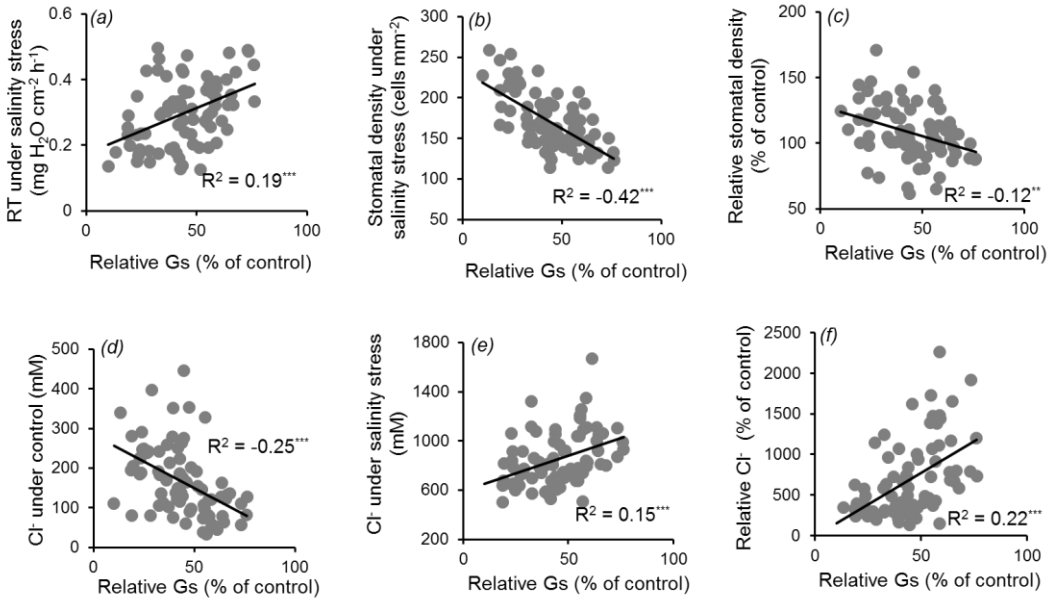


Fig. 5.12 (a) Correlation (Pearson's R^2 value) between the relative stomatal conductance (% of control) under salinity stress conditions and the residual transpiration under salinity stress conditions. (b) Correlation (Pearson's R^2 value) between the relative stomatal conductance (% of control) under salinity stress conditions and the stomatal density under salinity stress conditions. (c) Correlation (Pearson's R^2 value) between the relative stomatal conductance (% of control) under salinity stress conditions and the relative stomatal density (% of control) under salinity stress conditions. (d) Correlation (Pearson's R^2 value) between relative stomatal conductance (% of control) under salinity stress conditions and a leaf sap Cl^- content under control conditions. (e) Correlation (Pearson's R^2 value) between the relative stomatal conductance (% of control) under salinity stress conditions and a leaf sap Cl^- content under salinity stress conditions. (f) Correlation (Pearson's R^2 value) between the relative stomatal conductance (% control) under salinity stress conditions and the relative leaf sap Cl^- (% of control) under salinity stress conditions. Significant differences for a two-tailed test are indicated: *, P < 0.05; **, P < 0.01; ***, P < 0.001.

To find the most important traits that made major contributions to salt tolerance, a linear regression analysis was conducted as described in the methods. The preferred model retained the predictor RT under control conditions, and RT and

Na^+ under saline conditions. The model explained 22% of the variation in the outcome. Tolerant genotypes showed generally higher RT under control conditions but lower RT under salinity stress and lower Na^+ content under salinity stress (Fig. 5.13).

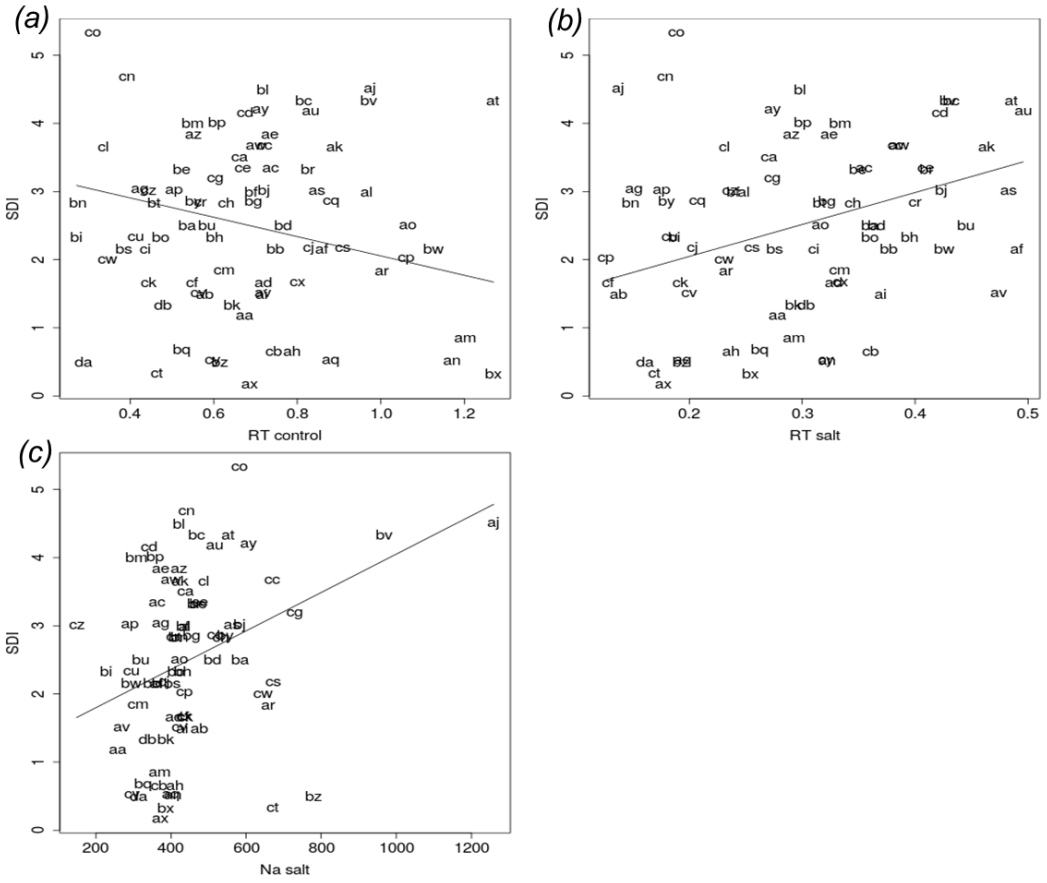


Fig. 5.13 A fitted regression model. Shown is the observed SDI, labelled by genotypes, against the three predictors in the fitted regression. Separate plots are shown for the three predictors including (a) RT control, (b) RT salt and (c) Na salt in the fitted regression model, each plot against one predictor. Also shown in each plot is the predicted mean regression line. Each regression line is calculated by holding the other two predictors at their mean value. Key to genotypes: aa=93-3143 C60; ab=AC Burman; ac=Aizao3; ad=Barque73; ae=Boa Fe; af=Brindabella; ag=Carmen; ah=Cevada de 2 ordens; ai=cevada Preta; aj=CI-4196; ak=CI-8826; al=Clipper; am=CM72; an=CPI71284-48; ao=CXHKSL; ap=DASH; aq=Dayton; ar=DYSYH; as=Flagship; at=Franklin; au=Gairdner; av=Gebeina; aw=Haruna Nijo; ax=Honen; ay=HOR12522; az=HOR12779; ba=HOR13437; bb=HOR13447; bc=HOR1448; bd=HOR2410; be=HOR3870; bf=HOR4055; bg=HOR8847; bh=HOR8851; bi=HU93-045; bj=Karin; bk=Keel; bl=KINU NIJO6; bm=Lixi143; bn=Macquarie;

bo=Mundah; bp=NN; bq=Numar; br=RGZLL; bs=Sahara; bt=Schooner; bu=Skiff; bv=Svanhals; bw=SYR01; bx=TAM407227; by=TF026; bz=TX9425; ca=Unicorn; cb=WA12908; cc=WA12915; cd=WA12916; ce=WA12918; cf=WA12924; cg=WA12927; ch=WA12930; ci=WA12931; cj=WA12937; ck=WA12949; cl=Westminister; cm=Xiaojiang; cn=Yan89110; co=Yan90260; cp=Yerong; cq=YF374; cr=Yiwu Erleng; cs=YPSLDM; ct=YSM1; cu=YSM3; cv=YU6472; cw=YUQS; cx=YWHKSL; cy=YYXT; cz=Zhepi 2; da=ZUG293; db=ZUG403

We used recursive partitioning on the SDI with candidate predictors being chlorophyll, residual transpiration, stomatal density, stomatal conductance, osmolality, Na^+, K^+, and Cl^- (ratios of saline to control). As shown in Fig. 5.14, only two predictors were selected, osmolality and residual transpiration. The salinity damage index was least when the ratio of saline to control for osmolality was below 1.73, slightly higher when between 1.73 and 3.53, and much higher values when was above 3.53. In the middle range of the osmolality ratio, the SDI had lower values when the ratio of saline to control of residual transpiration was below 0.29, and higher when above. All the comparisons were significant at the 0.01 level or below (Fig. 5.14).

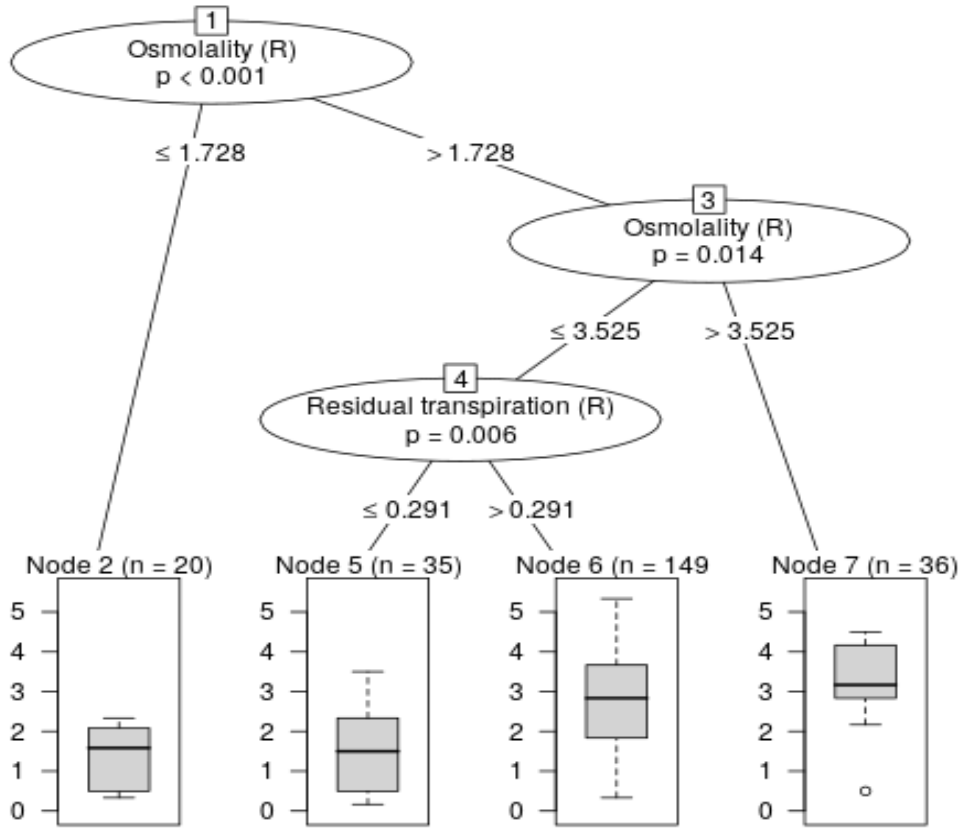


Fig. 5.14 The conditional inference tree for SDI. Shown are the results of the recursive partitioning. Only predictors that were included in the analysis appear. Each node represents a division of the data into two significantly different groups and the relevant *P* values are shown in each node. Cut points are shown in the edges below each node. The terminal nodes are represented using boxplots that summarise the distribution of data in each group. The sample size (n) for each group is given above the node. Each boxplot has a central box that indicates the range of the data between the 25th and 75th percentiles of the data, a horizontal line indicating the median, whiskers extending to 1.5 times the interquartile distance, and circles to indicate outliers.

5.4 Discussion

5.4.1 Genetic diversity of salt tolerance in barley

A large number of barley genotypes was used in this study, collected from different origin and habitat including winter v. spring, feed v. malt, six rowed v. two-rowed, awned v. awnless grown under high salinity conditions (300 mM

NaCl for 4 weeks) under glasshouse conditions. A significant variation of physiological responses was observed amongst the genotypes. Varietal difference for physiological responses under moderate to high salinity stress conditions has been reported in several studies under glasshouse conditions (Chen et al., 2007; Zhu et al., 2015). Therefore, such wide genetic diversity of barley physiological responses may open good prospects for barley breeding for salt tolerance.

5.4.2 Salinity tolerance in barley does not correlate with stomatal conductance

Stomatal limitation of photosynthesis is one of the main factors affecting plant growth under saline conditions. Reduction of Gs under salinity stress generally occurs as a result of a stomatal closure (Chaves et al., 2009). Previous studies have shown a positive relation between the Gs and salinity stress tolerance under moderate salinity exposures (James et al., 2008; Jiang et al., 2006). However, in this work no significant correlation was found between the Gs and salinity damage index in plants grown at much higher (300 mM NaCl for 4 weeks) salinity exposure (Fig. 5.9e). Thus, it appears that plants might adapt two different strategies while dealing with osmotic component of salt stress. When salinity levels are not high, tolerant varieties manage to maintain stomata open, assimilating more CO_2 and thus maintaining higher biomass. Under more severe salinity exposures, this strategy may compromise plant water status. Here, non-stomatal limitation of photosynthesis becomes central, and varieties capable to maintain higher chlorophyll content (Fig. 5.9b) display a better performance. The latter process seems to be causally related to the ability of tolerant genotypes to prevent hyper-accumulation of Na^+ in leaves (Fig. 5.9c). The ability of tolerant genotypes to efficiently sequester Na^+ in mesophyll cell vacuoles (Wu et al., 2015) and their capacity for preventing excessive ROS formation (Bose et al., 2014) may be also essential to confer this trait.

5.4.3 Residual transpiration is a component of a salinity tolerance mechanism

A set of physiological, anatomical and morphological adaptations ensures plants' ability to maintain WUE, to enable their survival and reproduction, even under adverse environmental conditions. During severe stress conditions, plants reduce stomatal transpiration near to zero by the stomatal closure. Under such

circumstances, plant performance depends on the efficacy of the cuticular (residual) transpiration barrier. By limiting RT from leaf surface, plant can increase the WUE under stress conditions (Franks et al., 2015). In our study, the RT of all genotypes was reduced under salinity stress conditions (Table 5.1). However, plant salt damage index correlated positively with the RT (Fig. 5.10a and 5.10b) indicating that tolerant genotypes were more efficient in reducing RT under stress conditions. These observations are consistent with previous reports on cereals under drought stress conditions, with 20% lower RT was recorded in barley under water stress conditions than the well irrigated conditions (González and Ayerbe, 2010). Similarly, it has been observed that wheat genotypes having lower RT adapted and performed better under water stress conditions (David, 2010). Thus, it appears that under water limiting conditions, reducing RT may be an important determinant of WUE and a potential mechanism for improving plant performance. The broad range of genetic variability in the relative changes in RT reported in this study (Fig. 5.2b) make it possible to select contrasting varieties and create DH lines, following QTL mapping of this trait.

Amongst other factors, the above reduction in the RT may be due to increased accumulation of cuticular wax on the surface of leaf, because the wax layer is a fundamental water transport-limiting barrier of the cuticle, especially when stomata are fully closed under stress conditions. Earlier studies showed the existence of a negative correlation between the total amounts of cuticular wax, particularly primary alcohol, and the RT in barley (Hasanuzzaman et al., 2017). Many studies have shown that drought stress increases the total amount of cuticular wax deposition with increasing cuticle thickness and changes composition of cuticular wax in different plant species (see details Xue et al., 2017). Under water deficit conditions, plants increased the total amount of cuticular wax per unit area of leaf by 80%; this was accompanied by 49% increases in the cuticle thickness compared with controls. The deposition of the total cuticular wax in response to the severity of drought stress could be regulated by different 'waxy' genes and improved the drought tolerance and adaptation. It has been documented that many such genes are involved in cuticular wax biosynthesis and accumulation in response to drought stress in different plant species (Xue et al., 2017; Yu et al., 2017). It was shown that the *Oswsl1, Osgl1-2*

and Osgl1-1/Oswsl2 mutants of rice that have reduced wax load were more sensitive to drought stress (Islam et al., 2009; Mao et al., 2012; Qin et al., 2011; Zhou et al., 2013). An overexpression of *WINI/SHNI, WXP1, OsWR1, ZmGL1, ZmGL15, ZmFDH1* and *ZmFAE1* genes enhanced higher accumulation of cuticular wax in *Arabidopsis*, alfalfa, rice and maize under drought stress conditions and improved drought tolerance (Aharoni et al., 2004; Wang et al., 2017; Yu et al., 2017). Since salinity stress is often referred to as a physiological drought, the tolerant barley genotypes may be depositing more cuticular wax on the leaf surface than the sensitive genotypes, leading to decreased rates of water loss from the leaf surface when stomata are closed, with a consequent increase in their WUE under salinity stress conditions.

A significant correlation between the RT and relative changes in Cl^- content in the leaf sap was found (Fig. 5.10d) while no such correlation was found for Na^+ content (Fig. 5.10e). This could be due to the fact that the plants increased their ability of Na^+ retention in the root and shoot to prevent its accumulation to toxic levels in the leaf and favoured the uptake and transport of Cl^-, whose concentration in the leaf was increased in this study. These results suggest that Na^+ delivery to the shoot is largely uncoupled from the transpirational stream, and plants were able to selectively control ion transfer to the shoot. The possible explanation may be related to the thermodynamics of the xylem ion loading. From this point of view, Cl^- loading into the xylem should be a passive (channel-mediated) process while xylem Na^+ loading may require involvement of the secondary active Na^+ transport systems such as SOS1 or CCC (see Shabala et al., 2013 and Zhu et al., 2017 for detailed arguments and supporting evidence). The overall accumulation of overall Cl^- was higher than that of Na^+ under both control and stress conditions suggesting higher preference of barley for Cl^- for the osmotic adjustment purposes. Previous researches have also reported higher permeability of Cl^- than Na^+ under normal and salinity stress conditions in different plants (Chakraborty et al., 2016; García and Medina, 2013).

5.4.4 Stomatal density under salinity stress

Stomata are the primary portals for the exchange of water and CO_2 for photosynthesis, which play a major role in CO_2 assimilation and ultimately

contribute to increase plant biomass and yield. Stomata are highly sensitive to environmental stress conditions and may change their number and distributions. In our study, SD decreased in only 1/3 and increased in the same number of genotypes (Fig. 5.3b). Among them, most of the tolerant (having lower salinity damage index) barley genotypes increased their SD under 300 mM NaCl conditions for 4 weeks (Table 5.1). The relative changes (% of control) of stomatal density of salt treated plants negatively correlated with the salinity damage index (Fig. 5.10f) indicating the importance of increasing stomatal density under higher salinity stress conditions as an adaptive tool to optimise the water use efficiency. These observations are consistent with previous study on barley suggesting that increasing SD positively correlated with plant salt tolerance (Zhu et al., 2015). This could be explained by the fact that tolerant barley genotypes' subsidiary and epidermal cells decreased their size during stomata formation under salinity stress thus increasing SD whereas sensitive genotypes were less efficient to do this. It has been suggested that stomatal development is interconnected by a 'passive dilution' mechanism in which SD are co-regulated by the size of epidermal cell (Murphy et al., 2017). Another reason for change in SD may be via developmental reprogramming. Stomatal density is controlled by a complex network of negative regulators in the epidermis such as *EPEDERMAL PATTERNING FACTORS (EPF1*, *EPF2)* and positive factors produced in the mesophyll known as *STOMAGEN* or *EPFL9* (Lawson and Blatt, 2014). Activation of these signalling peptides with membrane receptors *TMM* with other negative regulators *SDD1* and a linkage with several transcription factors like *SPCH*, *MUTA* and *FAMA* determine the stomatal pattern, number and size. It has been reported that stomatal mutants *sdd1-1* and *tmm1* of *Arabidopsis thaliana* grown under control conditions increased their SD over their wild types and SD were proportionally correlated with Gs (Vráblová et al., 2017). On the other hand, a higher CO_2 assimilation rate was found in *sdd1* mutant than their wild *Arabidopsis* as a result of higher Gs due to increase in SD (Lawson et al., 2014). The overexpression of STOMAGEN leads to 2 to 3-fold increase in SD, result in a 30% increase in photosynthetic CO_2 assimilation due to more CO_2 diffusion into leaf (Tanaka et al., 2013). Thus, increasing SD in salt-tolerant barley genotypes under high salinity stress condition could be an optimal strategy to maximise CO_2

assimilation. Interestingly, the overexpression of *HvEPF1* in monocot barley genotypes significantly reduced their SD and exhibited substantially increased drought tolerance and WUE under water withhold conditions without decreasing in grain yield (Hughes et al., 2017).

Interestingly, higher SD has resulted in higher leaf osmolality under both control and salinity stress conditions (Fig. 5.11a and 5.11b). SD is proportional to overall Gs per surface area; thus, higher SD supposes to deliver more Na^+ to the shoot via the transpiration stream to be used for osmotic adjustment purpose. The increasing osmolality is also a consequence of higher Na^+ and Cl^- accumulation. A significant positive correlation was found between increases in leaf sap osmolality and accumulation of leaf sap Na^+ (Zhu et al., 2017). For this reason, substantial amount of Na^+ and Cl^- may be used as the energetically cheaper osmolytes to keep proper turgor pressure in cells, allowing more K^+ in leaf for important metabolic processes.

K^+ is one of the most essential inorganic ions that play an important role in regulating stomatal movements. Salinity stress reduces stomatal conductance leading to reduced transpiration and photosynthesis. Interestingly, stomatal density significantly correlated with K^+ accumulation in the leaf under all conditions in our study (Fig. 5.11d, 5.11e and 5.11f). The plausible explanation for this fact could be that plant accumulated more K^+ in the leaf to maintain turgor pressure of stomatal guard cells to keep open their numerous stomata per unit area of leaf both under control and salinity stress conditions.

5.4.5 Changes in the stomatal conductance under salinity stress

Under salinity stress conditions, Gs decreased in all genotypes compared to control conditions (Table 5.1). As we mentioned earlier, no significant association was found between Gs and salinity damage index (Fig. 5.9e). However, higher relative (% of control) Gs values correlated significantly with higher RT under salinity conditions suggesting that water may transpire via closed stomata. Higher relative (% of control) Gs values were associated (Fig. 5.12b and 5.12c) with the lower SD under salinity stress conditions, suggesting a compensation mechanism. This can be explained by the fact that lower numbers but widely opened stomata

have the same capacity of CO_2 assimilation as higher number but partially opened stomata. Generally, plant adjusts to salinity stress by reducing leaf size to prevent stomata closure (Munns and Tester, 2008). Leaf area reduction also accompanied to a higher Gs per unit of leaf surface area by increasing SD in barley (Zhu et al., 2015). However, this appears not to be the case of halophytes. Salinity stress causes a significant (about 30%) decrease the number of stomata per unit leaf area (e.g. stomatal density) in quinoa suggested that less number of stomata widely open contributes to a higher stomatal density to fix more CO_2 (Shabala et al., 2012; Shabala et al., 2013).

5.5 Conclusions

Barley plants showed a range of morphological and physiological changes and employed different mechanisms and strategies to fight against the multifaceted effects of salinity. A significant reduction was observed in the residual transpiration in all the barley genotypes; tolerant genotypes were more superior in doing this, thus reducing water loss under salinity stress conditions. The residual transpiration negatively correlated with the stomatal density suggesting that the number of stomatal pores per unit area of leaf determined the transpiration loss through the cuticle of leaf surface. This trait could be targeted in breeding programmes to improve salinity stress tolerance in barley (and other species), to meet the demand of increasing food production.

5.6 References

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Chapter 6. Understanding physiological and morphological traits contributing to drought tolerance in barley[5]

Abstract

Drought stress is a major limiting factor for crop production in the arid and semi-arid regions. To increase the crop productivity, it is important to identify the desirable morphological and physiological traits that confer growth and yield potential under drought stress, to be incorporated into breeding programs. Accordingly, we screened eighty barley (*Hordeum vulgare* L.) genotypes collected from different geographical locations contrasting in drought stress tolerance and quantified a range of physiological and agronomical indices in glasshouse trails. The experiment was conducted in large soil tank subjected to drought treatment in eighty barley genotypes at three leaf stage and gradually brought to severe drought by withholding irrigation for 30 days under glasshouse conditions. Also, root length of the same genotypes was measured from stress-affected plants growing hydroponically. Drought tolerance was scored 30 days after the drought stress commenced based on the degree of the leaf wilting, fresh and dry biomass and relative water content. These characteristics were related to stomatal conductance, stomatal density, residual transpiration and leaf sap Na, K, Cl contents measured in control (irrigated) plants. Responses to drought stress differed significantly among the genotypes. The overall drought tolerance was significantly correlated with relative water content, stomatal conductance and leaf Na^+ and K^+ contents. No significant correlations between drought tolerance and root length of 6-day-old seedling, stomatal density, residual transpiration and leaf sap Cl^- content were found. Taking together, these results suggest that drought tolerant genotypes have lower stomatal conductance, and lower water content, Na^+, K^+ and Cl^- contents in their tissue under control conditions than the drought

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sensitive ones. These traits make them more resilient to the forthcoming drought stress.

6.1 Introduction

Drought is arguably the most hostile of all natural hazards, affecting plant growth and development and resulting in a major reduction in crop production globally. Global warming is expected to increase the frequency and intensity of drought in the twenty-first century (Pachauri et al., 2014). It has been predicted that climate change, causing high temperatures and scarce rainfall, will create increasingly extreme and prolonged drought from 1-3 % of the land for the present day to 30 % by the 2090s (Burke et al., 2006). Increasing drought occurrence affects the world's best food producing regions and jeopardises the world grain reserves and world food security, as a result of less frequent and uneven distribution of rainfall, increased evaporation or both (Cook et al., 2014; Dai, 2013). Increasing drought is projected to result in significant (over 75 %) losses in agricultural production worldwide, costing approximately $23.5 billion per year (FAO, 2015). This is especially worrisome given the need to increase, not just maintain, crop yields by up to 70 % to feed over 9.7 billion people by 2050 (Godfray et al., 2010). Thus, developing plant genotypes with higher drought tolerance and better adaptation is urgently needed.

The above development of drought-tolerant crops is complicated by the lack of highly tolerant genetic resources and the complexity in morphological, physiological and genetic traits conferring plant adaptation to drought. It is therefore important to identify the genetic resources and key morphological, physiological and molecular traits associated with improved survival to understand the mechanisms of drought tolerance in crops. Plant sensitivity to drought is determined by a range of important physiological and morphological parameters such as root length, photosynthesis rate, plant phenology, stomatal and non-stomatal (residual) transpiration, relative water content, and water use efficiency (Negin and Moshelion, 2017). Drought stress severely reduces plant growth and development by reduction of turgor pressure, cell elongation and expansion due to the osmotic stress (Farooq et al., 2009). Plants have developed multiple mechanisms through integrated morphological and physiological

responses to survive under drought stress conditions such as deep root system (Chloupek et al., 2010), manipulation of stomata (Franks et al., 2015), deposition of cuticular wax or cutinisation on leaf surface (Srivastava and Wiesenberg, 2018), leaf rolling (Zhang et al., 2018), increasing leaf thickness and succulence (Oliveira et al., 2018), osmotic adjustment through organic and inorganic compatible solutes (Turner, 2017). Among these adaptive mechanisms, stomatal and residual transpiration control and root system development are essential to the survival of plants under drought stress conditions. However, plant phenotyping under stress conditions is often a challenging task. For example, drought affected plants have their stomata closed, making measurements of gas exchange characteristics complicated. Similarly, phenotyping of root traits is hardly feasible under field conditions, and taking stomata imprints to quantify stomatal density is also impossible, due to the fact that all leaves are wilted and distorted. Thus, there is a strong need for simple and convenient physiological proxies that can be easily measured under control conditions and that can be used as reliable markers to predict plant drought responses.

Many previous studies have found a positive correlation between stomatal conductance (Gs) and grain yield under optimum growth conditions (Roche, 2015). On the other hand, high Gs under water deficit conditions can be detrimental due to the unwanted water loss from the leaf surface (Ouyang et al., 2017). The closure of stomata is an early response to drought stress that is associated with the high water use efficiency (WUE) (Hepworth et al., 2015). As Gs decreases photosynthetic rate is reduced due to the decreased inflow of CO_2 into the leaf. Stomata regulate the fluxes of both CO_2 uptake and water loss by plants. Moreover, Gs is often correlated positively with stomatal density (SD). Several studies showed that it is possible to improve drought tolerance and increase WUE by manipulating the SD on a leaf surface (Hughes et al., 2017). Stomatal development is regulated by the epidermal patterning factors (*EPF1*, *EPF2*) in the epidermis and *STOMAGEN* or *EPFL9* in the mesophyll. It has been documented that overexpression of *EPF1* and *EPF2* in *Arabidopsis* (Hara et al., 2009), poplar (Wang et al., 2016), and tobacco (Yu et al., 2008) mutants reduced stomatal density resulting in an improved WUE. In chapter 3 we demonstrated that drought tolerant barley genotypes contained lower SD than the sensitive

genotypes under optimum growth conditions (irrigated) and showed a negative correlation with drought tolerance index (Hasanuzzaman et al., 2017b). However, under drought stress conditions, when stomata are closed to minimise transpiration losses, a substantial amount of water may escape from the epidermis between stomata, termed residual transpiration (RT). RT varied among the barley genotypes being 20% higher under irrigated conditions than under drought conditions (González and Ayerbe, 2010). Under drought stress conditions, however, the balance between stomatal and residual transpirations is shifted and the latter could be as high as 28% of the stomatal transpiration (Boyer, 2000). Thus, reducing RT could be a potentially important mechanism to improve drought stress tolerance in plants. Tolerant genotypes of barley could decrease RT more than sensitive genotypes under drought stress conditions when stomata are closed or partially closed. This could be a survival capacity of plants under drought stress conditions, as it helps to conserve more water.

Roots are the first organs to sense drought stress and have been proposed as an important avenue of research to improve crop adaptation for their regulation of water availability to drought stress. Screening root traits at early stages of plant development could be a proxy trait at mature stages under drought stress (Comas et al., 2013). It is commonly assumed that the deeper and more prolific root systems are the key traits for maximizing water uptake: deeper root systems are able to forage more water and nutrients from the soil profile and improve performance under drought. However, the root length paradigm was questioned recently (Nippert and Holdo, 2015). Root growth increases relatively to shoot growth to acquire more water under drought stress conditions. Root metabolic costs would be significantly increased with increasing root length under drought stress. Genotypes having superior capacity to gain more water at reduced metabolic cost of root tissue would have greater productivity under water deficit conditions. So, selection of plant genotypes for long root systems regardless of the costs to the plant could actually decrease the yield. Root phenotypes that can reduce the metabolic cost of soil exploration could be promising and underexploited tools toward the development of crops with greater acquisition of water under drought stress conditions (Lynch, 2015).

In this work, we aimed to fill the above gaps in our knowledge by answering three specific questions: (1) do the stomatal density and stomatal conductance of barley genotypes grown under control conditions correlate with drought tolerance? (2) Does the residual transpiration of well-irrigated plants correlate with drought tolerance? (3) Is the root length an important component of the drought tolerance mechanism in barley?

6.2 Materials and Methods

6.2.1 Plant materials and growth conditions

Seeds of eighty barley genotypes (*Hordeum vulgare* L.) were obtained from the Australian Winter Cereal Collection and China and multiplied in the field at Tasmanian Institute of Agriculture (TIA) facilities in Launceston. Seeds were sown at 10 mm depth in 432 L (1.2 × 0.6 × 0.6 m) Poly (vinyl) chloride (PVC) tanks using the same amount of soil mixture with slow releasing mixed fertilizers in each tank in a glasshouse at the Mount Pleasant Laboratory facilities in Launceston, Australia. The experiment was conducted in 2015 (September – December) under controlled glasshouse condition (day length 14 h; average day/night temperatures 25/15°C; relative humidity 65%). After germination, barley seedlings were thinned to four uniform and healthy plants for each variety in each tank. Drought was imposed on 15-day-old seedlings (3-leaf stage) after germination. Plants were gradually brought to severe drought by withholding irrigation for 30 days. The experiment was conducted as a complete random design (CRD) with each tank as a replication. Thus, the experiment was carried out with four replications for each cultivar for each of drought and control treatments. Control plants were grown in normal irrigated condition in the soil tank under control glasshouse conditions.

6.2.2 Measurement of fresh and dry biomass and relative water content

Shoot fresh (*FW*) and dry (*DW*) weight were measured for control (irrigated) and drought stress conditions. To determine FW, plant samples were harvested between 09.30 -11.30 hours and weighed immediately after harvesting using a digital balance. Samples were then dried for 72h at 60°C in a drying oven and re-

weighed for determining DW. Relative water content (RWC) was estimated by using the following formula:

$$\%\text{RWC} = \frac{FW-DW}{FW} \times 100$$

6.2.3 Stomatal conductance measurement

Stomatal conductance was measured for control plants. Gs was measured in the fully expanded leaf using a steady state diffusion leaf porometer (model SC-1, Decagon, Australia). Leaves of intermediate position were used for measurements under constant light (artificial light of 900-1000 µmol m^{-2} s^{-1}) and temperature controlled glasshouse conditions between 9.30 and 16.00 h. Three replicates with four plants for each cultivar were measured.

6.2.4 Residual transpiration measurement

Three fully expanded leaves at an intermediate position from each genotype from control plants were selected for sampling. The leaves were excised and sealed with a vacuum grease on the cut end immediately. Collected leaves were then immediately transported to the laboratory and placed in the dark room at 20 ± 1°C and 50% relative humidity for stomata closure. Fresh weights were measured by an electronic balance immediately after excision of leaves. The leaves were then weighed at 2, 4 and 6 hour intervals. The leaves were then placed in dry oven at 60°C for 24h after which the dry weight was measured. The residual transpiration was measured and calculated as described in our previous publications (Hasanuzzaman et al., 2017a).

6.2.5 Stomatal density measurement

Stomatal density in barley leaves was quantified from leaf imprints from the leaves of the intermediate position grown under control conditions. For this an abaxial surface of the middle portion of a leaf was covered with a thin layer of a nail polish. Once dried, the imprints were peeled off using fine forceps, placed onto a microscope slide and covered with a cover slip. Imprints were examined microscopically at 20x magnification. The number of stomata was counted from each field of view and stomatal density (number of stomata per surface area) was

calculated. The sample size for each genotype was 18 (three fields of view × two imprints × three replications).

6.2.6 Na^+, K^+ and Cl^- measurements

Na^+, K^+ and Cl^- contents of the intermediate leaf were measured in control plants. For the determination of Na^+ and K^+ contents, leaf samples were put in 1.5mL Eppendorf tubes and frozen at -20°C overnight. Defrosted leaf samples were then squeezed to extract sap using a pointed glass rod. An amount of 100µL of the collected leaf sap was mixed with 20 mL of distilled water (200-fold dilution) and the mixture was evaluated using a flame photometer (MODEL PFP7 Flame photometer; Jenway, Felsted, Dunmow, Essex, UK). Chloride content in the leaf sap was measured using Cl^- selective microelectrodes by Microelectrode Ion Flux Estimation (MIFE) technique (Shabala et al., 2005; Shabala et al., 1997). Electrodes were calibrated in a set of Cl^- standards (250, 500, 1000µM for control plants) and positioned in a small chamber containing diluted leaf sap. The data were recorded using MIFE CHART software (Shabala et al., 1997) for at least 5min and Cl^- concentration was determined by taking the mean value of each measurement (Chakraborty et al., 2016).

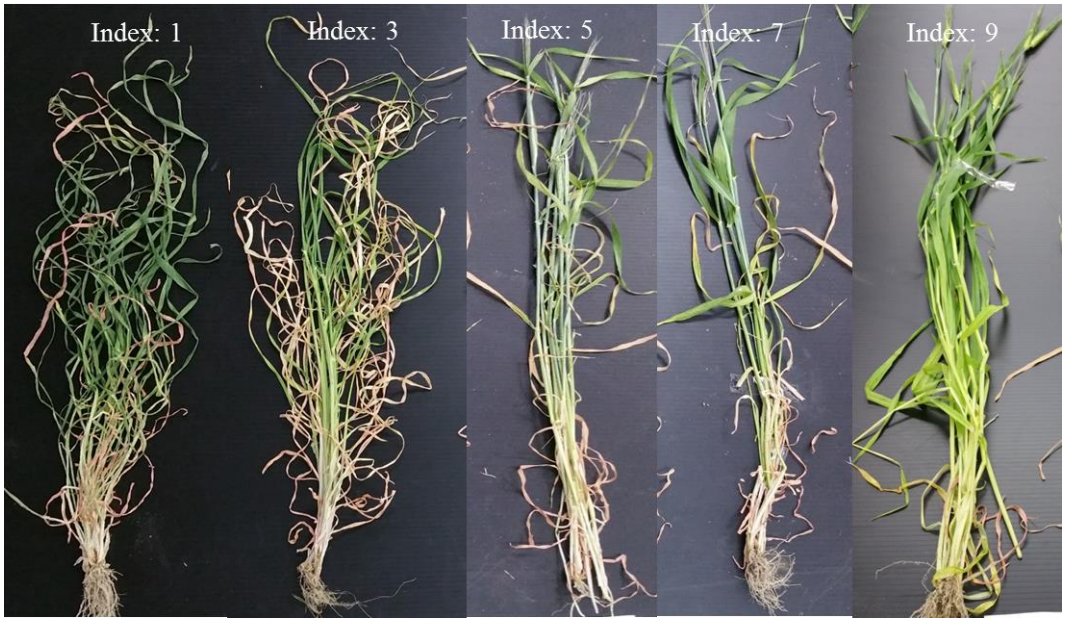
6.2.7 Drought stress tolerance index

After 30 days of withholding irrigation, the extent of the leaf injury was scored for each plant and ranked on 1 to 9 scales (1 = completely dead plants; 3 = more than 75 % of all leaves dried; 5 = more than 25 % of all leaves dried; 7 = about ¼ of the leaf length is dry; 9 = no damage symptoms) (Suppl. Fig. 6.1). The average values of four replications were used for quantitative estimation of drought tolerance. The drought tolerance index estimated as per above scale were used to determine the effectiveness of different morphological and physiological parameters in screening for drought tolerance.

6.2.8 Seedling test

All eighty barley genotypes were screened for root length under control conditions in hydroponics experiments. Fifteen seeds of each genotype were germinated in a two-layer wet paper towel inserted into a plastic pot containing small amount of distilled water. Seeds were grown in dark condition at 25°C.

After six days, the root length of the germinated seeds was recorded and correlated with drought stress tolerance index. For each of the genotypes, 10 seedlings from three biological replicates were analysed.



Suppl. Fig. 6.1 Visual assessment of drought tolerance in barley by using leaf injury scoring index 30 days after withholding water in a "big tank" under glasshouse conditions.

6.2.9 Statistical analysis

Data were analysed using IBM SPSS Statistics 21 (IBM corp. Armonk, NY, USA). All results are given as means ± SE. The significance of the correlations between different parameters was determined by bivariate correlations based on Pearson's correlation (two-tailed).

6.3 Results

6.3.1 Effect of drought stress on biomass production and relative water content

The effect of severe drought stress on plant performance was evaluated by analysis of the changes in the FW, DW and RWC after 30 days of withholding irrigation. About 3-fold variation was found in the FW among the genotypes under optimum growth conditions, with FW values ranging between 5.2 ± 0.2 g and 18.0 ± 0.5 g (Fig. 6.1a). FW significantly decreased in all genotypes 30 days

after withholding irrigation, bringing it into 0.70 ± 0.02 g and 4.4 ± 0.1 g range (Fig. 6.1b). The relative changes (% of control) of the FW of drought-affected plants showed a variation ranging from 10.0% for the genotype WA12931 (highest reduction) to 38.6% for the genotype Unicorn (Fig. 6.1c).

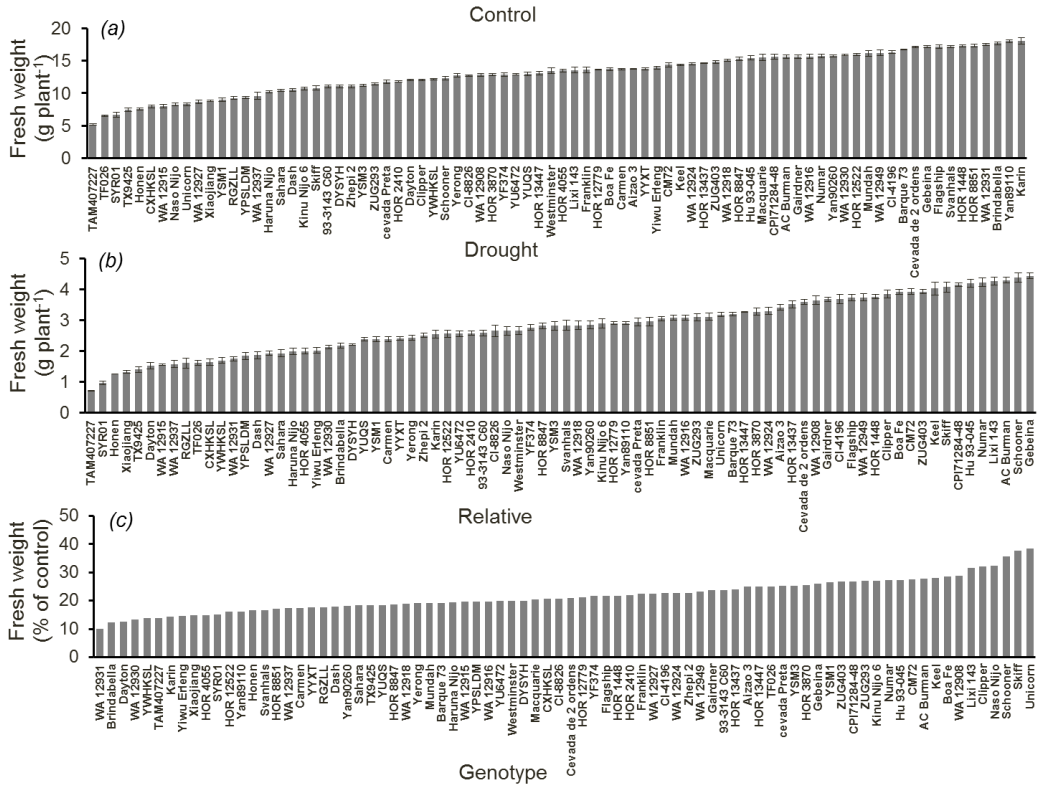


Fig. 6.1 Fresh weight (FW) of 80 barley genotypes under (a) control conditions and (b) drought stress (30 days after withholding irrigation) conditions. Data are means ± SE, *n* = 12. (c) Relative FW in drought-affected plants (expressed as a percentage of control).

Nearly a 5-fold variation was measured in DW among the genotypes under normal growth conditions, ranging from 0.6 ± 0.04 g for the genotype TAM407227 to 3.1 ± 0.1 g for WA12924 (Fig. 6.2a). Drought stress resulted in a significant reduction in the DW relative to well-watered controls in all genotypes tested ranging from 0.10 ± 0.02 g in the genotype TAM407227 to 1.8 ± 0.01 g in Gebeina (Fig. 6.2b). The relative values (% of control) of DW of drought-affected plants ranged between 26.2% (TAM407227) and 89.4% (HOR13437) (Fig. 6.2c).

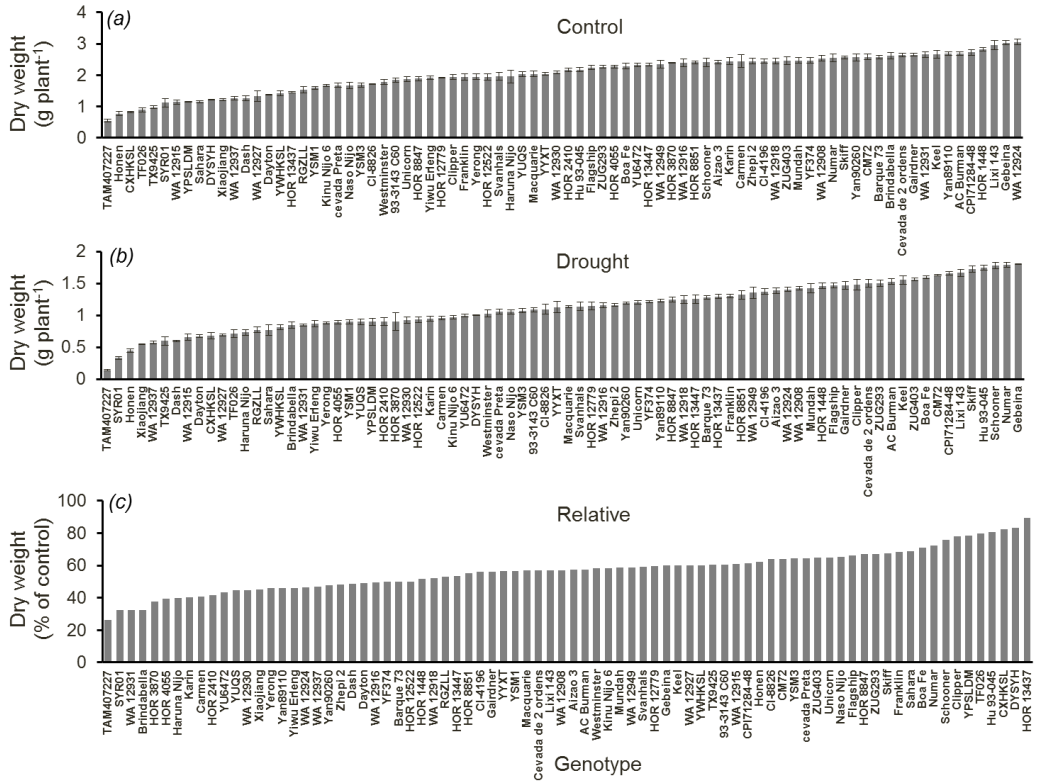


Fig. 6.2 Dry weight (DW) of 80 barley genotypes under (a) control conditions and (b) drought stress (30 days after withholding irrigation) conditions. Data are means ± SE, n = 15. (c) Relative DW of drought-affected plants (expressed as a percentage of control).

RWC ranged between 76.2 ± 0.3% in the genotype Skiff and 90.1 ± 0.2% in the genotype HOR13437 under normal irrigated conditions (Fig. 6.3a). A significant reduction was found in the RWC among all the genotypes 30 days after drought stress. This reduction ranged between 50.1 ± 1.8% (RGZLL) and 80.2 ± 1.8% (TAM407227) (Fig. 6.3b). The relative changes (% of control) of the RWC in drought stressed plants showed a genetic variability ranging from 57.9% in the genotype YWHKSL to 89.8% in TAM407227 (Fig. 6.3c).

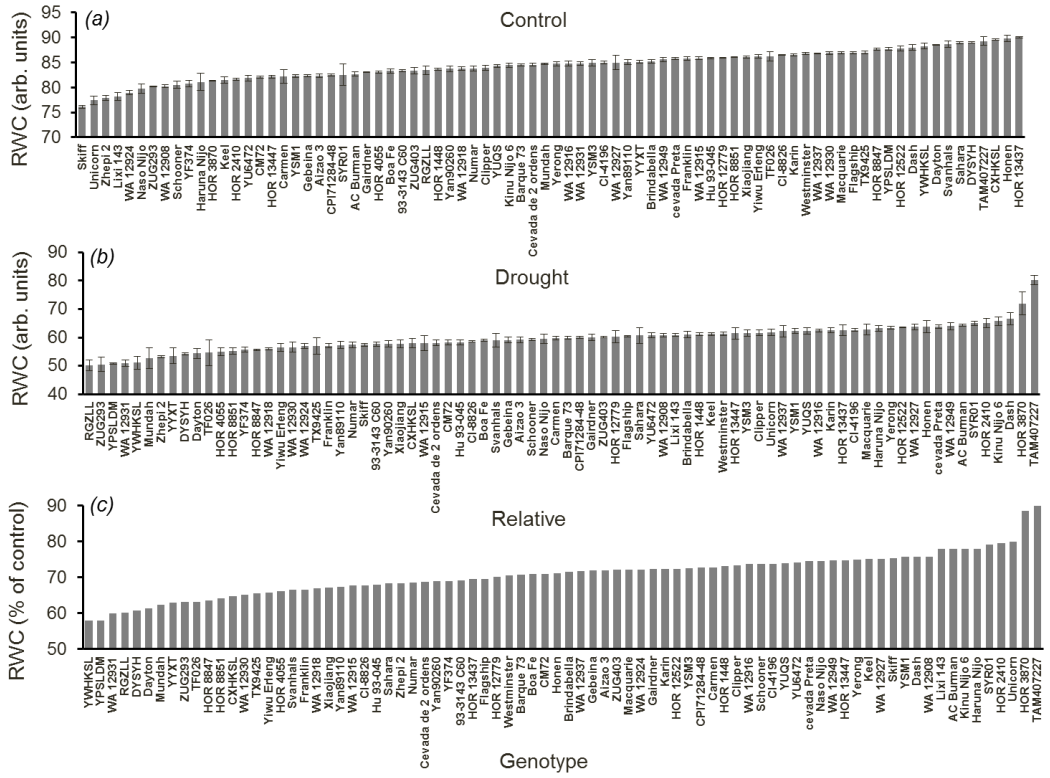


Fig. 6.3 Relative water content (RWC) of 80 barley genotypes under (a) control conditions and (b) drought stress (30 days after withholding irrigation) conditions. Data are means ± SE, n = 18. (c) Relative RWC of drought-affected plants (expressed as a percentage of control).

6.3.2 Correlation analysis

Eighty barley genotypes were grown under glasshouse conditions withholding the irrigation starting 15 days after the germination that continued for 30 days and scored according to the degree of leaf injury (Suppl. Fig. 6.1). Based on the leaf injury index, these genotypes were clustered into four groups: sensitive (injury score index 2.00 to 4.50), moderately sensitive (injury score index 4.50 to 6.00), moderately tolerant (injury score index 6.00 to 6.75) and tolerant (6.75 to 8) groups (Fig. 6.4a). To understand the contributions of different morphological and physiological traits to drought tolerance in barley, correlations between the drought tolerance index and different traits were calculated. The overall drought tolerance index was positively correlated with both absolute FW (R^2 = 0.21; P < 0.001) under control conditions, FW (R^2 = 0.37; P < 0.001) under drought stress conditions and relative changes (% to control) in FW values (R^2 = 0.32; P < 0.001) (Fig. 6.4b, 6.4c and 6.4d).

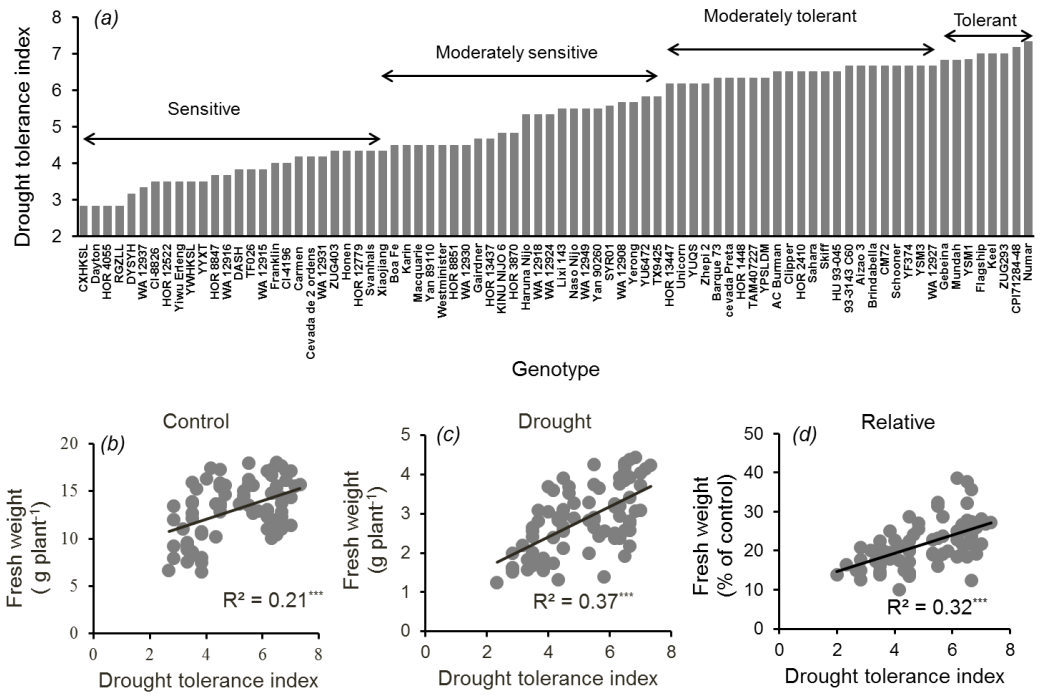


Fig. 6.4 (a) Eighty barley genotypes ranking according to their drought tolerance measured by the scoring index of leaf injury under drought stress (30 days after withholding irrigation) conditions. 1 indicates dead plants; 9 indicate no visual symptoms. (b) Correlation (Pearson's R^2 value) between fresh weight (FW) under control conditions and the drought tolerance index. (c) Correlation (Pearson's R^2 value) between fresh weight (FW) under drought stress conditions and the drought tolerance index. (d) Correlation (Pearson's R^2 value) between FW (% to control) under drought stress conditions and the drought tolerance index. *P < 0.05; **P < 0.01; ***P < 0.001 indicate significant differences by a two-tailed test.

Significant positive correlations were found between both absolute DW under irrigated (R^2 = 0.28; P < 0.001) and drought-grown plants (R^2 = 0.35; P < 0.001) and the relative (% to control) changes in the DW values (R^2 = 0.13; P < 0.01) potentially explaining the extent of higher biomass production in tolerant genotypes (Fig. 6.5a, 6.5b and 6.5c). RWC of control plants was negatively (R^2 = - 0.31; P < 0.001) correlated with the drought tolerance index (Fig. 6.6a). No correlation (P > 0.03) was found between RWC under drought stress conditions and the drought tolerance index (Fig. 6.6b). However, relative (% to control) changes of RWC were positively correlated (R^2 = 0.13; P < 0.001) with drought tolerance index suggesting that tolerant genotypes naturally contain less water in their shoots but keep it better under drought stress conditions (Fig. 6.6c).

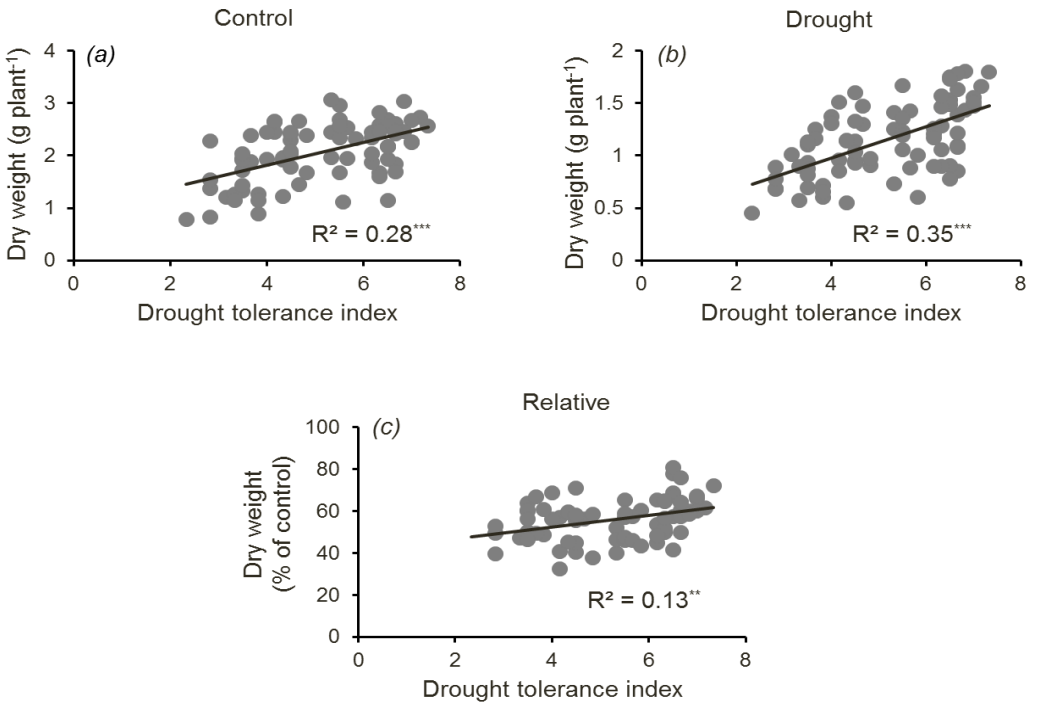


Fig. 6.5 (a) Correlation (Pearson's R^2 value) between dry weight (DW) under irrigated conditions and the drought tolerance index. (b) Correlation (Pearson's R^2 value) between DW under drought stress conditions and the drought tolerance index. (c) Correlation (Pearson's R^2 value) between DW (% to control) under drought stress conditions and the drought tolerance index. *P < 0.05; **P < 0.01; ***P < 0.001 indicate significant differences by a two-tailed test.

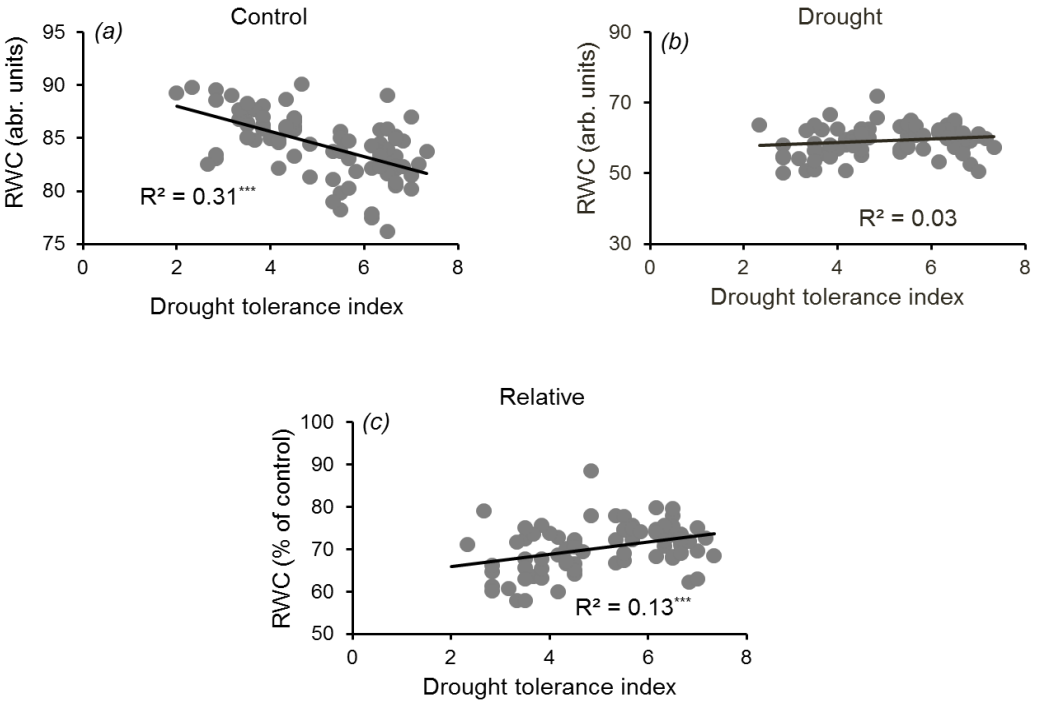


Fig. 6.6 (a) Correlation (Pearson's R^2 value) between relative water content (RWC) under control conditions and the drought tolerance index. (b) Correlation (Pearson's R^2 value) between RWC under drought stress conditions and the drought tolerance index. (c) Correlation (Pearson's R^2 value) between RWC (% to control) under drought stress conditions and the drought tolerance index. *P < 0.05; **P < 0.01; ***P < 0.001 indicate significant differences by a two-tailed test.

Root length of seedlings grown under control conditions was not significantly (R^2 = 0.02; P > 0.05) correlated with the drought tolerance index (Fig. 6.7a). A significant negative correlation (R^2 = - 0.24; P < 0.001) was found between Gs under control conditions and drought tolerance index (Fig. 6.7b). There was no strong correlation (R^2 = 0.07; P > 0.05 and R^2 = 0.02; P > 0.05, respectively) between either RT or SD in control plants with the drought tolerance index (Fig. 6.7c and 6.7d).

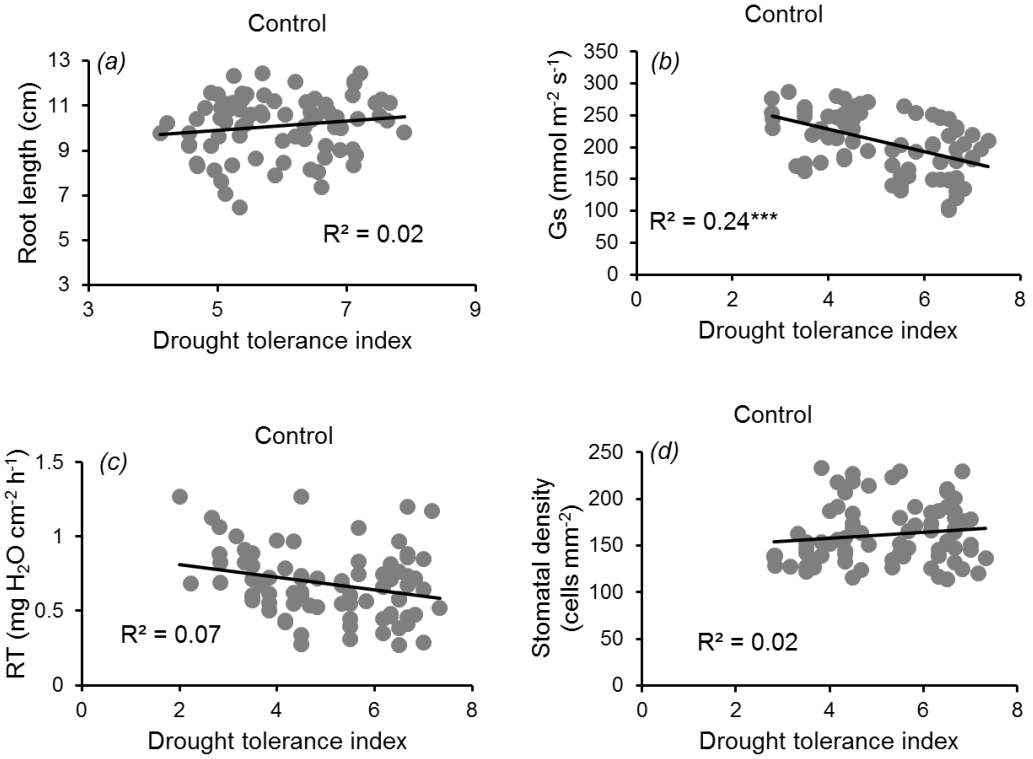


Fig. 6.7 (a) Correlation (Pearson's R^2 value) between root length under control conditions and the drought tolerance index. (b) Correlation (Pearson's R^2 value) between stomatal conductance under control conditions and the drought tolerance index. (c) Correlation (Pearson's R^2 value) between the residual transpiration under control conditions and the drought tolerance index. (d) Correlation (Pearson's R^2 value) between stomatal

density under irrigated conditions and the drought tolerance index. **P* < 0.05; ***P* < 0.01; ****P* < 0.001 indicate significant differences by a two-tailed test.

A significant negative correlation (R^2 = - 0.23; P < 0.001) was found between K^+ content in the leaf sap of control plants and the drought tolerance index. K^+ content in the leaf sap also significantly (R^2 = 0.23; P < 0.001) correlated with stomatal conductance under control conditions (Fig. 6.8b). Na^+ content in the leaf sap of control plants was significantly (R^2 = - 0.17; P < 0.001) correlated with the drought tolerance index (Fig. 6.8c). A significant negative correlation (R^2 = - 0.12; P < 0.01) was found between the drought tolerance index and Cl^- content in the leaf sap (Fig. 6.8d).

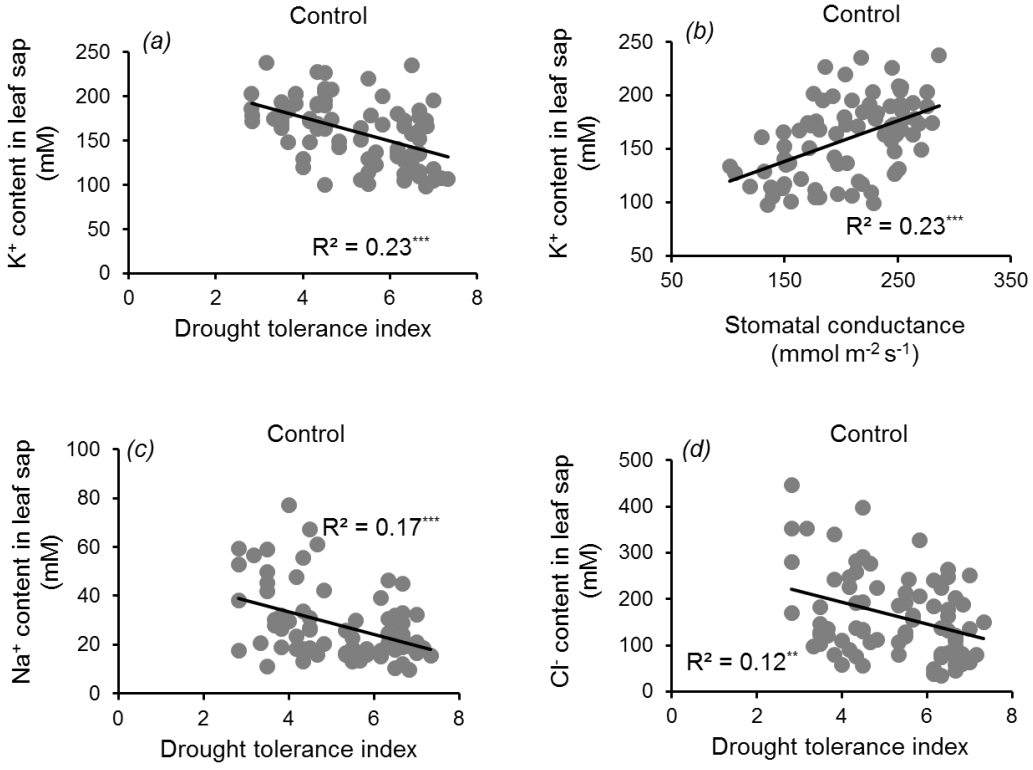


Fig. 6.8 (a) Correlation (Pearson's R^2 value) between K^+ content of leaf sap under control conditions and the drought tolerance index. (b) Correlation (Pearson's R^2 value) between K^+ content of leaf sap and stomatal conductance under control conditions. (c) Correlation (Pearson's R^2 value) between Na^+ content of leaf sap under control conditions and the drought tolerance index. (d) Correlation (Pearson's R^2 value) between Cl^- content under irrigated conditions and the drought tolerance index. **P* < 0.05; ***P* < 0.01; ****P* < 0.001 indicate significant differences by a two-tailed test.

6.4 Discussion

6.4.1 Genotypic variations in barley under drought stress

Eighty barley genotypes were used in this study, collected from different origins and habitats including winter, spring, feed, malt, six rowed, two-rowed, awned and awnless varieties. Significant variations in agronomical, morphological and physiological responses were observed among all the genotypes in response to drought stress. Of all 80 accessions, 26 (32%) genotypes were classified as highly sensitive, 23 (29%) genotypes were moderately sensitive, 23 (29%) were moderately tolerant, and 8 (10%) were classified as tolerant. These eight genotypes are Numar, CPI71284-48, ZUG293, Keel, Flagship, YSM1, Mundah and Gebeina. Most of these varieties are also known to be salt tolerant (Zhu et al., 2015). This is consistent with the notion of salinity stress being often referred to as a physiological drought and may be explained by the common signalling pathways shared by two stresses, including stomata regulation by stress-induced increase in ABA content or osmotic adjustment in root and shoot tissues (Munns, 2011; Chen and Jiang, 2010; Hong et al., 2013). Therefore, such wide genetic diversity of barley under water deficit conditions may open good prospects for barley breeding by using contrasting varieties to create DH population for QTL mapping, to develop drought stress tolerant barley germplasm.

6.4.2 Relative changes of FW, DW and RWC under drought stress

Biomass production occurs through cell division and cell elongation and involves a complex interaction of genetic, biochemical and physiological processes. In the present study, drought stress showed a significant effect on whole-plant fresh and dry weight, with both FW and DW correlating positively with the drought tolerance index (Fig. 6.4 and Fig. 6.5). The reduction of biomass production may be related to the reduction in the leaf area production. Drought stress inhibits meristematic activity by reducing cell division, cell elongation and expansion, resulting in decreased leaf area and plant growth (Avramova, 2016). Photosynthesis per unit leaf area and net assimilation rate are reduced because of stomata closure under drought stress (Chaves et al., 2009; Cornic, 2000). Biomass production depends on the stomatal behaviour under drought stress conditions as photosynthetic rates are linked to stomatal conductance. Under drought stress

conditions, the sensitive genotypes adopted a "survival" strategy by early stomatal closure which minimized CO_2 uptake and reduced photosynthesis, resulting in growth arrest and contributing to the reduced biomass production (Zhao et al., 2014). On the other hand, tolerant genotypes may redistribute resources from older leaves to young tissues by shedding old leaves thus helping the young leaves remain green and turgid by continuous water uptake (Schippers et al., 2015). Consequently, tolerant plants keep their stomata open (at least partially) and have the photosynthetic capacity under drought stress conditions, ensuring continuous growth, although at a reduced rate.

The RWC of shoot is an important physiological trait directly related to soil water. In our study, a significant negative correlation (Fig. 6.6a) found between the drought tolerance index and relative water content under control conditions indicates that drought tolerant varieties had naturally less water in their shoots. In contrast, relative water content was reduced under stress conditions but did not correlate with the drought tolerance index (Fig. 6.6b). At the same time, the relative change in RWC was less in tolerant genotypes and also positively correlated with drought tolerance index (Fig. 6.6c) indicating that tolerant genotypes initially contain less water in shoots but maintained hydration better under drought stress conditions. This could be explained by the fact that the tolerant genotypes naturally contain high level of organic osmolytes that are further upregulated under stress conditions (Swarcewicz et al., 2017; Templer et al., 2017). These osmolytes facilitate osmotic adjustment during water deficit conditions, and are used for plant defence and stress tolerance. Higher shoot water content under stress conditions is related to the capacity of plants to conserve water by efficient osmotic adjustment and reduced transpiration by stomatal closure (Blum, 2017). Therefore, when plants are subjected to drought stress, osmotic adjustments are effective to sustain tissue turgor, RWC and stomatal conductance at low leaf water potential (Boyer et al., 2008). The broad range of genetic variability in the relative changes in FW, DW and RWC reported in this study under drought stress make it possible to select contrasting genotypes and create DH population to map QTL for these traits.

6.4.3 Drought tolerance does not correlate with root length

More profuse and deeper root system has long been suggested as a major trait to improve crop adaptation under drought stress. There is a current trend in plant breeding to select the genotypes with longer root for drought tolerance (Comas et al., 2013; Paez-Garcia et al., 2015). In this study, we found no correlation between the root length of seedlings at 6 days of germination and the drought tolerance index (Fig. 6.7a) suggesting that longer roots play a limited role in barley adaptation to drought. Several structural and functional root and soil models investigated the cost-benefit relationship between different root traits in a wide range of environments in recent decades, suggested that root costs are considerable under drought stress (Lynch, 2013, 2015). Plants have a tendency to significantly increase root growth relative to shoot growth under drought stress. A greater root/shoot ratio indicates that each unit of leaf area has more non-photosynthetic tissue to sustain, which reduces overall plant growth rate. Producing long roots can protect against drought but it comes at a cost of carbon that could be used elsewhere. A number of studies have shown that the metabolic costs of soil exploration by root systems are substantial, and can exceed 50% of daily photosynthesis (Lambers et al., 2006). Crop genotypes with reduced metabolic costs of soil exploration would have improved water and nutrient acquisition. However, a steep-deep-cheap ideotype has been proposed to increase nutrient and water use efficiency for crop grown under certain conditions (Lynch, 2013). This model integrates root angles suitable for nitrate recovery, deep rooting for water and nutrient acquisition and reduction of root cortical cells through the development of aerenchyma to reduce the carbon cost of root maintenance (Lynch, 2015; Lynch et al., 2014; Mi et al., 2016; Zhu et al., 2010). To reduce the carbon cost of roots, another paradigm targets decreased root diameter, increased lateral root and root hair length, and increased root longevity over increased lateral root/root hair number and density (Meister et al., 2014; White et al., 2013). Therefore, the plant having root phenotypes that reduce the metabolic cost will have superior productivity, because it will retain more metabolic resources available for further resources acquisition, growth and reproduction.

6.4.4 Stomatal but not residual transpiration of control plant showed a strong correlation with the drought tolerance

Stomatal conductance is a the key parameter determining the limitation of photosynthesis, water use efficiency, growth and yield potential of barley under control and stress conditions (Jiang et al., 2006). A strong negative correlation between stomatal conductance under control conditions and the drought tolerance index has been found in this study (Fig. 6.7b) suggesting that tolerant genotypes have lower stomatal conductance in control conditions. Plants showing high Gs under control conditions will perform poorly under severe drought stress due to the hazardous water loss rate. On the other hand, a plant that always exhibits low Gs will be drought tolerant, but may not produce maximal yields under optimal conditions (Kholova et al., 2010). Stomata play the main role of gas exchange, both CO_2 uptake and water loss during transpiration, which ultimately contributes to carbon fixation in photosynthesis. Among other things, regulation of stomatal conductance depends on the distribution, size and stomatal density. However, in this study, no significant correlation was found between the drought tolerance index and stomatal density (Fig. 6.7d) under control conditions. The possible explanation was that under our experimental design (large tanks) where all plants were using a common water supply, adaptation of stomatal transpiration (as determined by traits such as stomatal density) becomes less important than other mechanisms. It was shown earlier that salt sensitive genotypes had higher stomatal density than the salt-tolerant and wild barley genotypes under normal growth conditions (Zhu et al., 2015). Also, it was reported that reduction in stomatal conductance via reduced stomatal density in *EPF2* overexpression in *Arabidopsis* plants increased their water use efficiency without affecting the photosynthesis (Franks et al., 2015). Hence, tolerant barley genotypes may transpire less water by lower stomatal conductance as they contained lower stomatal density under normal growth conditions and thus have better capacity to conserve water under drought stress conditions. Recently, it has been shown that the overexpression of *HvEPF1* gene in barley significantly reduced stomatal density and exhibited drought tolerance by increasing WUE without reduction of the grain yield (Hughes et al., 2017).

Residual transpiration is an important component of plant water balance, water use efficiency and stress reactivity, when stomata are closed (either fully or partially) under water deficit conditions. It has been reported that barley genotypes grown under drought stress conditions substantially reduced residual transpiration than the irrigated plants by increasing the amounts of cuticular wax on leaf surface, which ensured better and more stable yields (González and Ayerbe, 2010). Many previous studies reported that drought stress increases the total amount of cuticular wax by up to 3-fold and changes the composition of cuticular wax in different plants (Xue et al., 2017; Yeats and Rose, 2013), suggesting that the formation of cuticular wax on leaf surface under drought stress conditions acts as a component of non-stomatal transpiration barrier. This has been confirmed by our previous study that a negative correlation was found between cuticular wax load and residual transpiration (Hasanuzzaman et al., 2017a). However, in this study, no strong correlation was found between the drought tolerance index and residual transpiration of barley genotypes grown under well irrigated conditions (Fig. 6.7c) suggesting that cuticular wax deposition is not a constitutive but inducible trait.

6.4.5 Ionic relationship with drought tolerance

A significant negative correlation was observed between the drought tolerance index and K^+, Na^+ and Cl^- contents in the leaf sap under control conditions (Fig. 6.8a, 6.8b and 6.8c), indicating that drought tolerant genotypes had naturally less K^+, Na^+ and Cl^- in their tissues compared with their drought sensitive counterparts. Two possible explanations may be drawn here. First, K^+ uptake is used for the stomatal operation. This is confirmed by a significant positive correlation between K^+ content and stomatal conductance in our study (Fig. 6.8b). However, it appears that drought tolerant genotypes require less K^+ for stomatal operation. So when plants are having trouble of getting K^+ under drought conditions due to reduced transpiration, they are affected much less. Second, reduced K^+, Na^+ and Cl^- may suggest that plants rely more heavily on organic osmolytes for osmotic adjustment, to maintain tissue turgor. While it may come with yield penalties under control condition due to high energy cost of organic osmolytes productions (Shabala and Shabala, 2011; Zhu et al., 2015), many of these organic osmolytes have a dual role and also act as potent non-enzymatic antioxidants. It has been

suggested that proline and glycinebetaine, low molecular weight organic osmolytes, protect plants as a non-enzymatic antioxidants by detoxifying of ROS under stress conditions, thus protect membrane integrity, photosystem and stabilizing antioxidant enzymes (Hayat et al., 2012; Kumar et al., 2017; Ozden et al., 2009).

6.5 Conclusions

In conclusion, the study suggested that the tolerant barley genotypes contain less water in their tissues under control conditions and are more efficient in conserving water under drought stress. The latter trait is achieved by the more pronounced reduction in stomatal conductance. The stomatal density and residual transpiration of barley genotypes grown under control conditions were not correlated with drought tolerance, suggesting that these two traits are not constitutive but rather inducible. The drought tolerant genotypes had naturally lower Na^+, K^+ and Cl^- contents in their tissues compared with their drought sensitive counterparts and, thus, relied more on organic osmolytes for osmotic adjustment. These osmolytes most likely played a dual role protecting stressed plants against the oxidative stress imposed by the water deficit.

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Chapter 7. General Discussion and Conclusion

Salinity and drought stress are amongst the most important abiotic stresses reducing cultivable land and crop production worldwide. Consequently, it is urgent for a major breakthrough in crop breeding for salinity/drought tolerance to ensure the future food demand for increasing world population. Thus, there is a need to identify the genetic resources with higher tolerance, and to understand the mechanisms of salinity/drought tolerance in plants. Plant responses to both salinity and drought stress both create osmotic stress and cause reduction in plant growth and yield. Plant growth and development under osmotic stress conditions are often influenced by the multiple mechanisms, with the involvement of the morphological and physiological factors within the plant and the interaction between the plant and its growing environment. The complexity of plant salinity and drought tolerance, the lack of reliable and comprehensive screening methods, and the lack of a comprehensive understanding of the underlying morphological and physiological mechanisms of salinity and drought tolerance obstruct a further improvement in selecting and breeding for salt- and drought-tolerant crop species. Barley is a major cereal crop cultivated worldwide, and suffers large yield penalties from both salinity and drought stress in their growth habitants. However, the multiple complexities of osmotic stress obstruct the improvement of breeding true osmotic stress tolerance barley species. In light of the above, this study into whole-plant physiological and morphological responses to salinity and drought stress goes towards clarifying the mechanism of salt and drought tolerance in barley. The specific aim of this GRDC funded research were (i) to investigate the importance of the residual transpiration as a component of salinity tolerance mechanism; (ii) to reveal the role of cuticular waxes as a determinant of the residual transpiration; (iii) to evaluate the suitability of various physiological and morphological traits as a proxy for drought tolerance; (iv) to understand the selective physiological and morphological traits contributing to drought tolerance in large number of barley genotypes.

7.1 Residual transpiration is a component of salinity stress tolerance mechanism and cuticular waxes act as a determinant of the residual transpiration

The contribution of residual transpiration on overall salt stress tolerance in barley and the role of cuticular wax on residual transpiration were investigated in this study. RT and cuticular wax content were first measured in four barley cultivars contrasting in salinity tolerance at three different leaf positions (old, intermediate and young leaves) under control conditions. Then, eighty barley genotypes were screened under 300 mM NaCl conditions in a separate experiment to evaluate the genotypic variation and contribution of different physiological traits including RT towards salinity stress tolerance. Results suggested that old leaves transpired more water than young leaves for all four barley genotypes and RT was higher in salt tolerant genotypes than the sensitive genotypes under normal irrigated conditions. RT was negatively correlated with the total amount of cuticular wax and the component of cuticular wax primary alcohol revealed that cuticular wax represented as a major water barrier in the leaf surface. However, when eighty barley genotypes were investigated under higher saline conditions, it was found that tolerant barley genotypes reduced the RT and showed a significant negative correlation with the salinity tolerance (lower salinity damage index). Among the all above physiological trait that showed significant correlations with salinity damage scores, RT under control conditions, RT under salinity stress and Na^+ content under salinity stress had major contributions to salinity tolerance. The combination of these three physiological traits would be determined 20-28% of the phenotypic variation of salinity damage scores. Tolerant genotypes showed generally higher RT under control conditions but lower RT under salinity stress and lower Na^+ content under salinity stress. Therefore, it was observed that minimisation of RT is a fundamental mechanism by which plants could improve their water use efficiency under salinity stress conditions. In an ecophysiological context, previous studies in a diversity of plant species and environmental conditions assumed that residual transpiration is an important strategy to minimise unwanted water loss from the leaf surface to survive in a dehydrating environmental conditions (Schuster et al., 2017; Domínguez et al., 2017). However, if breeders are targeting genes conferring residual transpiration as a

component of salinity stress tolerance in barley, then the next logical step would be to undertaken QTL mapping of genes conferring of this trait using DH populations.

In this study, it was found that cuticular wax on barley leaves consisted mostly of very-long-chain fatty acids (VLCFAs) and their derivatives including primary alcohols, aldehydes, *n*-alkanes, benzoate esters, phytol related compounds, fatty acid methyl esters, β-diketones and alkylrescorcinols. This result showed consistency with previous studies on the cuticular wax analysis of the different plant species (Jetter et al., 2006; Yeats and Rose, 2013). Molecular analysis of wax-deficient mutants including *eceriferum (cer)*, *bloomless (bm)* and *glossy (gl)* with no cuticular wax has identified a large number of genes responsible for the biosynthesis, transport and regulation of cuticular wax in different plant species (Lee and Suh, 2015). Different 'waxy' genes including *CERs, CER6, WIN1/SHN1*, *KCS*, *OsWSL1*, *OSGL1-6, WAX2* have been well-recognised for the biosynthesis of cuticular wax and practically applied in improving stress tolerance in different crops (Xue et al., 2017). However, cuticular wax formation is a composite in nature. It is important to identify the role of each component of cuticular wax to water permeability across the cuticle (Fernández et al., 2017). It has been identified that the intracuticular VLCFAs as the main barrier of the water diffusion across the cuticle (Vogg et al., 2004) but recent studies suggested that epicuticular waxes also contributed to water movement in some cases (Jetter and Riederer, 2016). Therefore, it is important to use different modern technologies such as mass spectrometry imaging (MSI) for analysis of cuticular wax constituents; GWAS and CRISPR-CAS9 for genes identifications and gene-editing, respectively for exploiting natural and artificially induced genetic variability of cuticle related traits for breeding purpose to improve stress tolerance crops (Domínguez, 2017; Cohen et al., 2017; Petit et al., 2017).

7.2 Whole physiological responses and the prospects of F_v/F_m ratio for drought tolerance screening

In addition to the physiological and genetic complexity of the drought tolerance traits, the lack of convenient and reliable screening techniques also obstructs the progress in barley breeding. In view of this, six barley genotypes were screened

and quantified a range of physiological and morphological responses under drought stress and subsequent recovery. Drought stress was imposed at 20-day-old seedlings by withholding irrigation and brought to 10% soil water content and kept at that level by maintaining a fixed pot weight on a daily basis for 2 weeks and then rewatering the pots back to their full water holding capacity. Genotypes were evaluated by measuring transpiration rate, SPAD chlorophyll meter reading, dry biomass, shoot water content, chlorophyll fluorescence F_v/F_m ratio. Chlorophyll fluorescence F_v/F_m measurement at drought stress conditions and transpiration measurement at the recovery stage showed a strong correlation with drought tolerance. However, transpiration measurement is quite time-consuming for large scale screening, when thousands of leaf sample need to be analysed. Chlorophyll fluorescence measurements are rather simple, reliable, non-destructive and rapid (only a few seconds per leaf sample is required for F_v/F_m measurement from dark adapted leaves) (Oukarroum et al., 2009; Munns et al., 2010). Therefore, for a large scale drought screening of barley genotypes for breeding programme, the maximum quantum efficiency of light harvesting in PSII in dark-adapted leaves (F_v/F_m ratio) is likely to be the most efficient physiological parameter for screening plants for drought tolerance and could be used as a suitable proxy for screening. However, breeders would use this trait conferring the drought tolerance QTL mapping of genes using DH populations.

7.3 Physiological responses of barley and the role of stomatal density under higher salinity stress conditions

In the current study, a number of physiological parameters have been evaluated to potentially understand traits contributing to salinity tolerance using eighty barley genotypes under 300 mM NaCl in glasshouse conditions. These included chlorophyll content, stomatal conductance, stomatal density, leaf sap Na^+, K^+ and Cl^- concentration, and leaf sap osmolality. It was shown that the traits including leaf Na^+ content, stomatal density and osmolality following the 4-weeks treatment of 300 mM NaCl can be used as reliable selection criteria for the breeding of higher-salinity tolerant barley genotypes, because of lower leaf Na^+ content, osmolality, and higher stomatal density observed in tolerant genotypes. Stomatal conductance is not suitable for using as a selection criterion under higher salinity stress conditions, as it did not correlate with salinity tolerance. However, under

moderate salinity stress conditions, tolerant plant may manage to keep stomata open for photosynthesis, but this mechanism may compromise plant water status. Stomatal density could be a selection criterion for salinity tolerance, as salt-tolerant barley genotypes increased their leaf stomatal density under much higher salinity stress conditions. It has been documented that stomatal development, patterning, size and density are influenced by a complex network of different genes containing *SPEECHLESS (SPCH)*, *MUTE*, *FAMA*, *EPIDERMAL PATTERNING FACTOR (EPF)*, *STOMAGEN*, *STOMATAL DENSITY AND DISTRIBUTION1 (SDD1)*, *TOO MANY MOUTHS (TMM)*, *ERECTA LIKE (ERL)* and *YODA* (Lawson and Blatt, 2014; Peterson et al., 2010; Vatén and Bergmann, 2012). Consequently, alteration of specific genes determining stomatal patterning and distribution would be incorporated to progress breeding for salinity tolerance. *Arabidopsis*, the model plant, has been successfully used in the past decades to clarifying the basis molecular framework controlling stomatal development and pattering. The recent evolutionary developmental biology studies have showed that the core stomatal developmental genes are expressed in different stomatous species, and identified the core stomatal development pathway in more species with the help of genomic sequences and gene editing tools like CRISPR-CAS9 system (Chater et al., 2017; Qu et al., 2017). The basic hypothesis is that increasing or decreasing stomatal density would increase or decrease stomatal conductance, respectively. However, in our case, stomatal density correlated negatively with stomatal conductance suggesting that higher number of partially opened stomata per unit area of leaf may contributes to fix more CO_2 for photosynthesis under higher salinity stress conditions. Interestingly, stomatal density increased with increasing accumulation of K^+ content in leaf sap under higher exposure of salinity stress, indicating that tolerant genotypes are more capable of accumulating K^+ to maintain the turgor of guard cells of stomata to keep open their higher number of stomata per unit area of leaf. Another interesting observation was that reduction of residual transpiration is related to increasing stomatal density. Therefore, increasing the number of stomata per unit area of leaf could be targeted in plant breeding programme to increase the salinity tolerance in barley.

7.4 Physiological and morphological adaptations of barley under drought stress

In this study, eighty barley genotypes were exposed to drought stress by withholding irrigation to understand the physiological and morphological response in drought tolerance. These included fresh and dry biomass, relative water content, stomatal conductance, residual transpiration, stomatal density, leaf sap Na^+, K^+ and Cl^-, and leaf sap osmolality. The drought tolerance was evaluated by visual scoring of leaf injury. Root length of the same genotypes was evaluated to germinating seeds in paper rolls in a separate experiment. The results indicated that the drought tolerance genotypes had naturally lower stomatal conductance and lower tissue water content under control conditions, thereby using these mechanisms, plants may tissue water under drought stress conditions to survive. Another observation was that the drought tolerant genotypes had naturally less Na^+, K^+ and Cl^- ions in their tissues compared to the drought sensitive genotypes. Therefore, reduced uptake of Na^+, K^+ and Cl^- ions in plant tissues may indicate that tolerant genotypes rely more on organic osmolytes for osmotic adjustment under drought stress conditions rather than inorganic ions, as organic osmolytes play dual roles like compatible solutes and non-enzymatic antioxidants. The study suggested that, plants had a chance to adapt by other means rather than the manipulation of stomatal density under the slower but longer drought stress onset. Longer roots do not contribute to improve drought tolerance but other root phenotypes like profuse root hairs and lateral roots development may help to increase water absorption area and reduce metabolic carbon cost.

7.5 Potential use of barley genotypes against salinity and drought stress

This project has been funded by Grains Research and Development Corporation, GRDC, Australia to explore the tolerant barley genetic resources against both salinity and drought stress conditions. Among studied barley genotypes, collected from different geographical locations Numar, CPI71284-48, ZUG293, Keel, Flagship, TAM047227, TX9425, CM72, YSM1, Mundah and Gebeina were found to be the most drought and salinity tolerant. On the other hand, Franklin, Gairdner, Cl-4196, Yan90260, Yan89110, Haruna Nijo and Kino Nijo were found

to be the most drought and salinity sensitive genotypes. However, these barley genotypes could be used as a potential donor of both salinity and drought tolerant genes in breeding program on barely.

7.6 General conclusion and recommendations

In conclusion, the overall studies suggested that residual transpiration is a component of osmotic stress tolerance mechanism and minimization of residual transpiration under stress environmental conditions could be a promising way of improving water use efficiency. The deposition of total amounts of cuticular wax or cuticular wax components, specifically primary alcohol on leaf surface acts as a water barrier to protect non stomatal water loss across the leaf surface. Stress-tolerant genotypes can be developed by targeting metabolic pathways responsible for stress-induced increase in the accumulation of cuticular wax on leaf surface to protect the residual transpiration under stress environmental conditions. Increasing stomatal density could also be an adaptive tool to optimise water use efficiency under osmotic stress conditions. Targeted genetic modification of stomatal density could be a viable approach for the engineering of higher WUE in crops under stress conditions. Key features targeted by breeding should include both physiological and morphological traits under stresses and control conditions. Plants with less relative water content, lower stomatal density, stomatal conductance and lower Na^+, K^+ and Cl^- content in their tissue under irrigated conditions showed better drought tolerance under drought stress conditions. The broad range of genetic variation in the relative changes of residual transpiration, stomatal density, and chlorophyll fluorescence reported in this study under both salinity and drought stress conditions make it possible to identify the contrasting genotypes and produce DH population for QTL mapping of these traits for development of osmotic stress-tolerant barley genotypes.

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